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PARAMECIUM AURELIA AND *PARAMECIUM CAUDATUM*.*

GARY N. CALKINS, PH.D.

AT the present time, when the subject of mutations and species is discussed on every hand, and when every eye is keenly on the alert for new evidence among animals and plants, a sudden transformation of one known species into another known species is of interest. Such an incident has recently come under my observation; a *Paramecium caudatum* became *P. aurelia*, and remained so for about 45 generations, when it reverted to *P. caudatum*. Apart from the facts of the change, which in itself is of obvious importance from the standpoint of cellular biology, the essential question to consider is whether these two species are sufficiently well defined to justify their separation. If not, then the experiments and the changes indicated have less bearing on the general problem of mutation than upon the problems of cell physiology. If they are sufficiently distinct, then we have in this incident an interesting case of mutation. I personally believe that the slight differences that distinguish the two types of Paramecium are not of specific value, and hold that *P. caudatum* should be regarded as a mere variant of *P. aurelia*.

Paramecium aurelia was the name given by Müller, in his general work on the Protozoa in 1773, to the ciliated organism which had been known as the "slipper animalcule." Several different species of Paramecium were created by Ehrenberg in 1838, and described in his work on the *Infusionsthierchen*. Most of these have been sifted out into other genera, and only three have remained, *P. bursaria*, *P. aurelia*, and *P. caudatum*. *Paramecium caudatum* and *P. aurelia* have been united into a single species by the majority of observers subsequent to Ehrenberg, on the ground that the differences upon which Ehrenberg had based his species were inadequate. The number of species was thus reduced to two, and the names used were *P. aurelia* and *P. bursaria*, the former having been given originally by Müller. Maupas, however, in 1887–89, and R. Hert-

* Received for publication March 17, 1906.

wig in 1889, discovered a difference in the two forms which appeared to have specific value, and since then the two species in question, *caudatum* and *aurelia*, have been generally accepted as "good" species.

Paramecium aurelia, according to Maupas, differs from *P. caudatum* in the following points: It is smaller (70 to 290 μ, as against 120 to 325 μ in *P. caudatum*); its posterior end is rounded, while *P. caudatum* has an attenuated end (hence *caudatum*); it has two small micronuclei (3 to 5 μ in diameter), while *P. caudatum* has but one (8 to 10 μ); in conjugation its macronucleus becomes "ribbon"-shaped at an earlier period than in *P. caudatum;* and after conjugation its cleavage nucleus gives rise to four corpuscles, whereas in *P. caudatum* there are eight. In deciding which of these forms to call *caudatum* and which *aurelia*, Maupas could not determine which type Müller had seen, and went back therefore only to Ehrenberg, who in naming *P. caudatum* had noted the attenuated posterior end. Hence it turns out that the more common form of Paramecium has become widely known as *P. caudatum*, while the less common form bears the original name *P. aurelia*. If the two are only variants of the same species, it follows from the rules of zoological nomenclature that the common and well-known name *Paramecium caudatum* must be given up and *P. aurelia* substituted. That this must be the case follows, as I believe, from the observations here described. In the following description the names *P. caudatum* and *P. aurelia* will be used for those variants of the organisms which agree with Maupas' specific characteristics.

On March 11, 1905, four pairs of conjugating *Paramecium caudatum* were isolated from a culture that had been running for some weeks in the laboratory. Each pair was confined in a hollow ground slide in a medium of hay infusion made the previous day by boiling a small quantity of hay in tap water. The usual period of conjugation is from 18 to 24 hours, and by the following day all of the pairs had separated, and the different individuals were swimming about freely in the hay infusion as apparently normal ex-conjugants. Each individual was isolated and fed on hay infusion, and each became the progenitor of a more or less extended line of Paramecia, the method followed being the same as that described

DIVISIONS OF EX-CONJUGANTS DURING THE FIRST 10 WEEKS AFTER SEPARATION.

	March																			April																		
	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
A	1	1	1	2	2	4	5	7	8	9	10	11	12	13	15	16	17	18	18	18	19	19	20	21	22	24	25	26	27	28	29	31	33	34	34	34	34
B	1	1	1	1	1	1	1	1	2	2	dead																										
C	1	1	1	dead																																	
D	1	lost																																			
E	1	1	1	1	1	1	1	1	1																												
F	1	1	1	1	1	1	1	1	1																												
G	1	1	2	2	3	4	5	6	7	8	9	11	12	13	15	16	17	18	19	20	21	21	22	23	24	26	27	28	29	31	34	35	37	38	39	40	41
H	1	1	1	2	2	2	2	3	3	3	3	3	dead																								
X	1	3	4	6	5	7	9	12	13	14	15	16	17	18	19	19	20	22	24	26	27	28	30	31	..	34	35	36	37	39	40	41	43	45	46	47	49

	April												May																								
	19	20	21	22	23	24	25	26	27	28	29	30	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	34	34	34	35	36	38	40	41	43	45	45	45	45	45	45	46	46	47	47	48	49	51	52	53	55	55	57	59	61	62	63	65	66	67	68	69
B																																				
C																																				
D																																				
E																																				
F																																				
G	42	43	45	46	48	49	51	53	55	57	57	59	60	61	62	63	64	65	67	68	70	71	73	74	76	77	79	81	83	84	85	86	87	90	92	92
H																																				
X	50	52	53	54	54	54	54	54	55	57	59	60	60	61	62	64	64	66	68	70	72	74	75	77	77	78	80	81	83	85	86	88	90	91	93	94

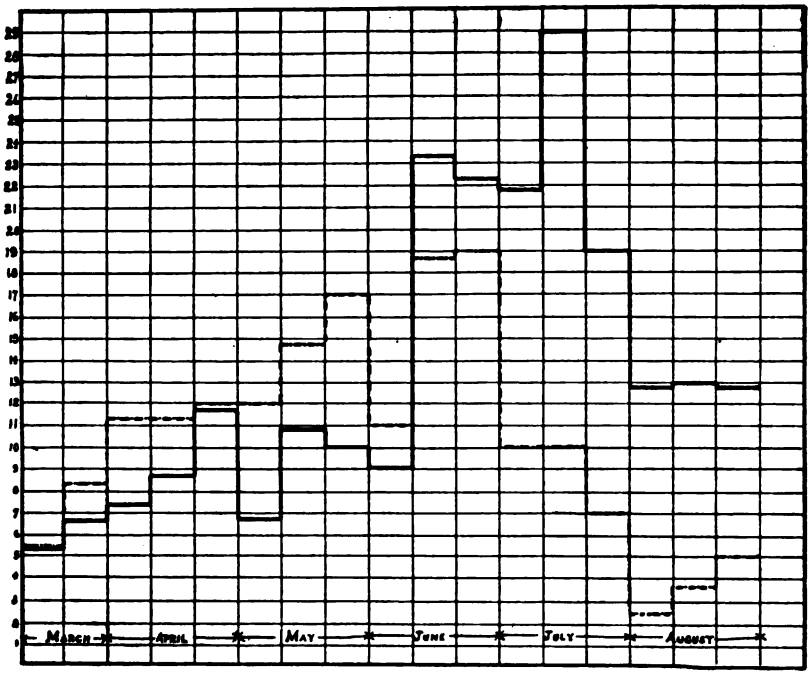
in a previous publication. As in previous work, the number of divisions was recorded each day for each of the ex-conjugants. In addition to the ex-conjugants, one individual from the original culture which had not conjugated, was isolated at the same time to serve as a sort of control. This line was designated X; the others, A–B, C–D, E–F, G–H, etc.

The early history of the division rate for all of these forms is given in the accompanying table.

The interest in the present paper centers in the first pair, A–B, for it was in this line of cultures that the *aurelia* form appeared. B died after a few days, but A recovered from the shock of reorganization and soon began to divide at a slow rate. As is the custom in such work, one of the daughter-cells from an early division was killed and stained, to determine if conjugation had been normally completed. The preparation shows that, instead of reorganizing with one micronucleus characteristic of *P. caudatum*, this individual had two, but otherwise appeared normal. From time to time after this individuals from this line of cultures were fixed and stained, and these preparations give a good history of the nuclear conditions at different periods. Some of these specimens are shown in photographs on Plate 1. Fig. 1 represents an individual in the third generation after conjugation, with four micronuclei (already divided for the ensuing generation) and the as yet incomplete macronucleus. Fig. 2 represents an individual in the 24th generation; Fig. 3, one in the 43d; Fig. 4, one in the 46th; Fig. 6 shows two micronuclei in the end stage of division, the daughter-halves are connected by a delicate thread not seen in the photograph; Fig. 7, finally, represents an individual in the 220th generation after complete recovery of the *P. caudatum* condition. The change from the one form to the other occurred during the first two weeks in May. Figs. 3, 4, and 5 represent three individuals from the same ancestor in the 43d generation. The first two individuals have each two micronuclei, the third has only one. All of these micronuclei show a marked increase in size over those shown in Fig. 2 for example, representing an individual in the 24th generation.

During the month of May and until three months after the culture was started, individuals appeared here and there with but one

micronucleus, while the majority of them killed at this time appeared with one of the micronuclei larger than the other. By the end of June none of the *P. aurelia* forms were to be found, and this culture, like the other cultures started at the same time (G and X), contained forms with only one micronucleus; *Paramecium aurelia* had become *P. caudatum* again. This occurred between the 45th and the 70th generations, and the effect of this change upon the vigor of the race is evident from the remarkable rise which the accompanying curve representing the relative division rate takes at this period (see curve).



Curves showing one nearly complete cycle, and the variations in vitality as measured by the division rate for ex-conjugants of the A series (solid line) and the G series (broken line). The ordinates represent the average number of divisions in 10-day periods. The rise in the A curve, as indicated in the first 10 days in June, marks the recovery of the uninucleate condition characteristic of *Paramecium caudatum*.

Maupas held that the two species of Paramecium can be readily distinguished by the characteristics given above. If we examine these characters in the light of the experience with cultures, we find that they cannot hold good. For example, the relative size of the

two forms which Maupas, and with him more recently Simpson, held to be a distinctive feature, loses all value when considered critically, while size relations in general are absolutely untrustworthy in settling questions of species. The variations which a species of Paramecium passes through under different conditions of vitality are so great, and last for such long periods, that no inference can be drawn from cell dimensions at any given time. Size depends, apparently, upon two factors—the relative vitality, and the rate of cell division—and these two may probably be merged into one, which may be called the potential of vitality. *Paramecium caudatum* under different conditions shows wide variations in size. When taken directly from the natural habitat, where food is not overabundant, they are large, measuring on the average 315 μ. The same forms cultivated on hay infusion, with its rich food content, multiply rapidly, and do not grow individually to the same size as the "wild" form. These measure on the average (18 individuals killed at different periods of the cycle) only 206 μ, with variations from 180 to 224 μ when the division rate was relatively high—two divisions per day. When the potential of vitality is nearly exhausted and the division rate is low, a similar small size is noticed; but at such a time it is obviously due to a different cause, probably a loss of metabolic energy. The same differences are noticed in *P. aurelia* at different periods of vitality, and the impossibility of considering size relations as of specific value is clearly established. During the first 45 generations of *P. aurelia* the division rate averaged only eight-tenths of a division per day, which is lower than that for the G and X lines, which averaged one and one-tenth divisions. Eighteen individuals killed at different periods of the culture were measured during these 45 generations, and the average length was 224 μ, with variations from 168 to 256 μ. After the loss of one of the micronuclei the division rate increased to the remarkable rate of 2.2 per day on the average for a period of four months, when vitality waned. During this period of rapid multiplication the size averaged only 178 μ with variations from 148 to 212 μ. At this period the organisms in culture would have been identified by any microscopist as *P. caudatum*, although more than 40 μ, on the average, smaller

than the one which would be classed as *P. aurelia*, while the latter, in turn, measured 90 μ less, on the average, than wild *P. caudatum*. It is quite apparent, therefore, that size cannot be taken as a diagnostic character in the present case; and this is, after all, only the application of a well-known principle in zoölogical taxonomy.

The pointed condition of the posterior end, also, in *P. caudatum* is likewise transitory, and may or may not be present in forms which agree in all other characters. Pointed Paramecium, if isolated, and the descendants watched for four or five generations, as I have done, will lose this characteristic and will become rounded and blunt at the posterior end (cf. Figs. 3, 4, and 7).

The sluggishness which Simpson advanced as a specific character of *P. aurelia* is purely a physiological condition, depending upon the vitality at a given time, and is as much characteristic of *P. caudatum* as of *P. aurelia*.

The breaking-up of the macronucleus at an earlier period in conjugation, which Maupas considered a diagnostic feature of *P. aurelia*, may also be due to physiological conditions. I cannot write definitely on this point, as I have had no experience with conjugating forms of *P. aurelia*. By itself it would not constitute a diagnostic characteristic of sufficient value to determine a species. So, too, the other characteristic feature of conjugating forms, named by Maupas, the number of corpuscles into which the fertilization micronucleus divides, would be dependent upon the number of micronuclei present, and would amount to the same thing in either case, if each of the two micronuclei of *P. aurelia* forms four micronuclei, as Maupas describes. The experience which I have described above of the presence of two micronuclei after conjugation in forms which had only one before, indicates that eight corpuscles characteristic of *P. caudatum* were also formed here, but were resolved into a binucleate instead of a uninucleate condition. This characteristic, therefore, cannot be termed specific.

Apart from the purely physiological characteristics which have little or no value in classification, there remains only the one specific feature to justify the attempt to separate *P. aurelia* and *P. caudatum*, viz., the presence of two and one micronuclei respectively. My experiments show that this, too, is inadequate, for *P. caudatum*

may become *P. aurelia*, and *P. aurelia* may in time lapse again into *P. caudatum*. It is to be inferred from this that the various forms which have been described as *P. aurelia* are in reality only "sports" of *P. caudatum*, and if such "sports" are unable to keep up by inheritance the characteristic structural differences which distinguish them from the ancestral form, although this is maintained for the long period of 45 generations, they cannot be considered a "good species." On the other hand, R. Hertwig gives some evidence, not conclusive, however, to show that *P. aurelia*, after conjugation, reorganizes with two micronuclei.

It must be admitted that one experience of this kind may be insufficient to throw out a species that has appeared to be so well established. It may be that my observation was made on a chance abnormality which paralleled *P. aurelia*, and that the real *P. aurelia* retains its integrity as a species. Personally, however, I do not believe it, and am reasonably confident that such abnormalities may be of frequent enough occurrence in nature to account for the numerous descriptions of *P. aurelia* that have been given. Forty generations is a long series for an abnormality to be transmitted, and the number of individuals represented by 2 to the 40th power (as my culture represents), allowing for natural loss through enemies, etc., would provide enough specimens with this abnormality to justify the belief that it is normal. On the other hand, it cannot be stated that *P. aurelia* is a well-established species. It is relatively rare in nature; its specific character has been contested by such eminent authorities as Bütschli, Engelmann, Balbiani, Stein, Koelliker, and Gruber; while Maupas and Hertwig succeeded in establishing it as a species only on the slender basis given above. My one experience with this culture is strong enough, as I believe, to reanimate the old skepticism, and to justify us in abandoning either *P. aurelia* or *P. caudatum*. The latter is the more recent name given by Ehrenberg, and according to the rules of priority must be replaced by *Paramecium aurelia*, the name applied by O. F. Müller to the "slipper animalcule."

The physiological features presented by this experiment give some interesting data upon the vitality and nuclear relations. The curve shows that a sudden rise in vigor accompanied the return

to the normal through the loss of one of the two nuclei. Although it is difficult to estimate accurately the relative volumes of nucleus and cell body, some idea of the relationship can be given by measuring the two dimensions of the surfaces exposed. The surface of the macro- or micronucleus obtained by multiplying the two dimensions exposed, have a measurable relation to the total surface of the organism, and in a rough way this relationship represents the relative volumes. Measured in this way, it was found that in resting, vegetative cells of *P. aurelia* the relation on the average of both micronuclei together to the whole organism is as 1:717 (or of the single micronucleus to total body as 1:1,434). In dividing cells this proportion rises to the ratio of 1:608 (or for the single micronucleus as 1:1,216). In the *P. caudatum* stage, after the loss of one micronucleus, the ratio of the micronucleus to the entire cell is expressed by the ratio of 1:648 for resting cells and 1:440 for dividing cells.

The macronucleus is generally considered to be the primary agent in constructive processes of the cell, and therefore of the first importance in considering the growth and division energy. In the *P. aurelia* phase, when the division rate was low, the average relation of the macronucleus to the whole body was as 1:43, with wide variations from 1:16 to 1:68. In the *P. caudatum* phase, with one micronucleus, the proportion of the macronucleus to the entire cell fell to 1:50 on the average, with variations from 1:39 to 1:82. The difference is not great, and may well fall within the limits of experimental error, and it is reasonable to infer that the physiological differences which are evident between the *aurelia* and *caudatum* forms do not owe their origin to the difference in the mass of macronuclear material. On *a priori* grounds it is reasonable to expect the more rapid multiplication in forms with the greater proportion of micronuclear material, and this is borne out in the experiments for it appears from the curve that during the period of abnormal micronuclei, when the smaller amount of micronuclear material was distributed in two nuclei, the organism was laboring under abnormal physiological conditions and had a lower division rate than when the normal uninucleate conditions were restored.

EXPLANATION OF PLATE I.

M = macronucleus.
m = micronucleus.

FIG. 1.—Ex-conjugant of the A series in the third generation after union. Four micronuclei can be seen to the right of the macronucleus, and, in addition, a relatively large unabsorbed fragment of the old macronucleus. The micronuclei are precociously divided for the fourth generation.

FIG. 2.—*Paramecium aurelia* in the 24th generation. The two micronuclei may be seen at the lower end of the macronucleus, one on each side.

FIG. 3.—*Paramecium aurelia* in the 43d generation. The two micronuclei are of relatively large size, and are dissociated from the macronucleus at the upper end of the animal (the two objects at the right of the macronucleus are foreign particles deposited on the organism).

FIG. 4.—*Paramecium aurelia* in the 46th generation. The two micronuclei are at the upper end of the macronucleus, and cannot be seen distinctly.

FIG. 5.—*Paramecium aurelia* in the *caudatum* phase at the 43d generation after union. The single micronucleus may be seen at the extreme right end of the nuclear material. It is noticeably larger than the micronuclei of the *aurelia* phase (compare Fig. 2). This specimen is one of the same lot as that represented by Fig. 3.

FIG. 6.—*Paramecium aurelia* in the 32d generation with macronucleus and micronuclei in division. Three of the micronuclei are shown with pointed ends; the fourth is out of focus and does not show.

FIG. 7.—*Paramecium caudatum* phase of *P. aurelia*, in the 220th generation. The relatively large micronucleus is dissociated from the macronucleus. This specimen was killed when the division rate was high (end of July; cf. curve).

PLATE I.

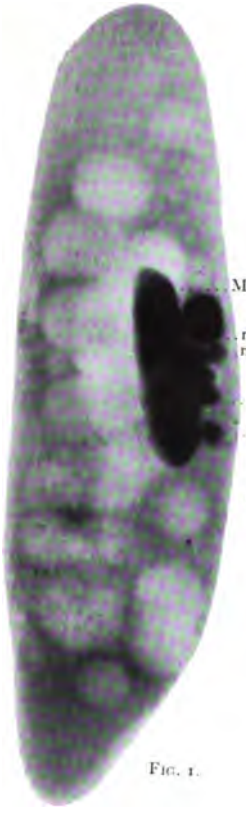


FIG. 1.

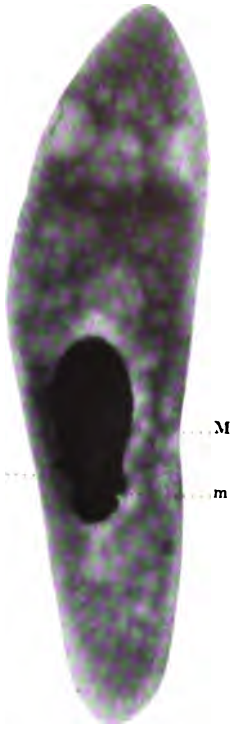


FIG 2.

FIG. 3.

FIG. 4.

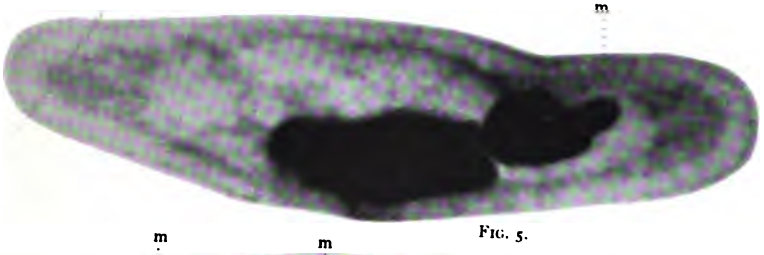


FIG. 5.

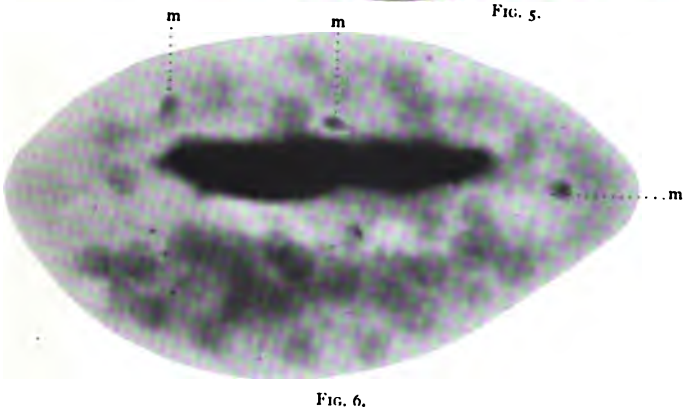


FIG. 6.

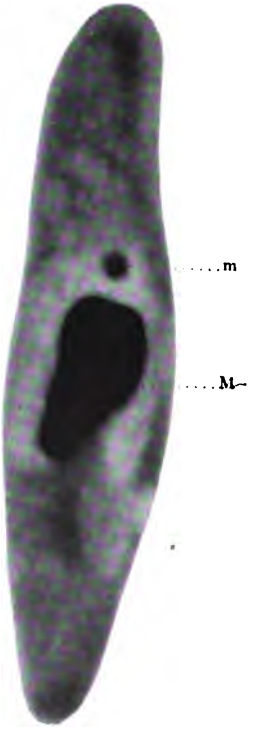


FIG. 7.

THE LIFE-HISTORY OF A COCHLIDIAN MOTH—*ADONETA BICAUDATA* DYAR.*

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THE larvæ of the Cochlididæ, or slug caterpillars, are especially interesting to the entomologist on account of their peculiar forms and considerable diversity of structure. The author has been interested in this family for twenty years, and has been so fortunate as to work out the life-histories of many of the species inhabiting the Atlantic coast region of North America, as well as a few foreign ones. The results have been published in a number of articles;[1-26] so it is not necessary to repeat here the general structural characters of these larvæ. They are highly specialized forms of Tineoidea or Microlepidoptera; that is, the larvæ are highly specialized. The moths have not shared in the complication of structure exhibited in their early stages. The family is spread throughout the world, and is doubtless of early origin in geologic time.

North of Florida and Texas, where peculiar forms occur, 26 species are at present known. Of these I have found the larvæ of 20, and have been especially interested to secure the remaining six, if possible. It was the search for one of these, *Monoleuca semifascia* Walker, which resulted in the knowledge of the life-history of *Adoneta bicaudata*, though not successful in its immediate object. In 1898 and 1899 I made collections of larvæ at Morris Plains, N. J., where it was said that *Monoleuca* had been taken many years ago, hoping that it might still be there. The larvæ were very small; but as nothing that looked to be the one sought appeared, they were turned over to Mr. L. H. Joutel, of New York. Later in the season Mr. Joutel called my attention to two larvæ which were new to him that he had noticed among his stock, and which he thought were from among those I had given him, or else came from Long Island. The new larva was allied to *Adoneta spinuloides* Herrich-Schaeffer, and we thought it might be the *Mono-*

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leuca, since that genus is allied to *Adoneta* as adult moths. We were not then successful in rearing the new larva. It is referred to in print[24] as *Monoleuca* (*?*), as I then thought it might be. Subsequently, in arranging the collections at the National Museum at Washington, I found an inflated larva of this new form, together with a bred female adult, prepared by Mr. A. Koebele, formerly of the United States Department of Agriculture. The adult was not *Monoleuca*, but a light-colored form of *Adoneta*, which I supposed must be Packard's *leucosigma*, though I could not be sure that the new larva properly belonged to it, not finding Mr. Koebele's notes. On the strength of this specimen, I listed *leucosigma* as a variety of spinuloides.[29] In 1903 and 1904 Mr. W. F. Fiske, of the Forestry Division of the Bureau of Entomology, was collecting at Tryon, N. C., and he turned up *Monoleuca semifascia* in numbers. At my request, Mr. Fiske secured eggs, but they all proved infertile. In the fall of 1904, therefore, I went to Tryon for the purpose of finding the *Monoleuca* larva. I was unsuccessful; but in the search obtained a number of larvæ of the new form of *Adoneta*. In the same season a number of adults were collected at Plummer's Island in the Potomac River, near Washington, by Messrs. August Busck, H. S. Barber, and E. A. Schwarz, and it became evident that I had before me an undescribed species of *Adoneta*, which was not *leucosigma* Packard. I therefore published a description of it,[30] associating with it the new larva.

The larvæ from Tryon resulted in several male and one female moths in July, 1905. The males emerged before the female, and, in flying in the cage, lost their legs, so that they quickly destroyed themselves by buzzing on the ground. When the female emerged, I had no living male left. I placed her in the cage at Rock Creek, near Massachusetts Avenue, in the District, but no male was attracted. The next night Mr. H. S. Barber kindly took the cage to Plummer's Island and remained there all night, but again without success. However, Mr. Barber took at light two males of this species, which he kept alive in glass tubes. One had lost its legs and was useless, but the other was in good condition and was placed in the cage. Mating occurred on the third night after emergence of the female, and good eggs were secured.

Adoneta bicaudata is remarkable for the lateness of emergence of the adults, late July and early August being the time of flight. One other species, *Isochaetes beutenmuelleri* Hy. Edwards, has similar habits and a similar distribution. Northern New Jersey and Staten Island N. Y.,[27] seem to be the limits of their range northward, and it is doubtful if they occur so far north in every season. Their lives as larvæ are as long as those of any other species, namely about eight weeks, so that they are liable to be destroyed by early frosts before maturing. After spinning their hard cocoons on the ground or among dead leaves, they are immune to cold, though pupation does not occur till the following spring. The species is, of course, strictly single-brooded.

The eggs are laid in groups of two to ten, overlapping, and placed on the backs of the leaves. They are usually deposited in low places, young red oak bushes in not too dense woods being the best situation. None were found on the higher branches. At Tryon, in 1904, all the larvæ taken were on small red oak trees. In 1905 I took a larva near Washington (Rosslyn, Va.), also on red oak, and a number at Tryon at the end of September on this tree, and also on other plants, white oak and *Ceanothus*. The little larvæ, on hatching, separate, having no tendency to be gregarious. Their first business is to molt, which they do within two days of hatching, without having fed during the first instar. In the second stage feeding begins. The young larvæ, up to the fifth or sixth stage, eat the lower epidermis and parenchyma of the leaf, cutting short, scattered, irregular channels of about the width of their bodies. They frequently change their feeding-place and pass readily from one leaf to another, but of course cannot leave the tree on which the eggs were deposited. When large enough, they begin to eat the whole leaf from the end or side inward, as the other species of Cochlidians do. The larvæ observed by me passed nine stages, though they can, no doubt, mature in eight under more favorable conditions. I was obliged to carry my larvæ to northern New York, where they were fed on yellow birch, oak not being available, and were subjected to the rigors of an Adirondack climate. When matured the larvæ change color slightly and leave the tree to spin their cocoons among dead leaves on the ground, wherein they pass the winter in the prepupal stage.

As compared with its nearest ally, *Adoneta spinuloides* H. S., the larva of *A. bicaudata* is narrower, less elevated at joint 5; the horns of joint 13 are long, predominant over those of joint 12, which exceed those of joint 10; the widenings of the dorsal purple band are all subequal, five widenings, each practically alike, no pushing out of the subdorsal horns of joints 6 and 7; caltropes reduced on the anterior side horns, joint 6, *et seq.*, only well developed on joints 12 and 13; the color of the dorsal band is bluer purple than *spinuloides*, less reddish or maroon.

DESCRIPTION OF THE SEVERAL STAGES.

Egg.—Elliptical, colorless, shining, flat, the surface faintly reticular. The yolk forms a slightly opaque central mass, leaving a transparent rim. On the leaf the eggs are shining and transparent, resembling drops of moisture. Their extreme flatness enables them to be laid overlapping like shingles. Size, 1.4×0.8×0.1 mm. The development of the embryo can be easily observed in eggs laid on glass. The shell is thin and transparent, merely a delicate skin. It is impossible to detach the eggs without destroying them. They hatch in 10 days.

Stage I.—Elliptical, the dorsum flattened, the sides oblique, the venter flat. Head small and weak, without hard chitinous covering; mandibles and palpi present, but reduced and weak; the mandibles with four equal blunt teeth incapable of feeding, colorless. A subdorsal and a lateral row of processes or "horns," one on a segment, the subdorsals on joints 3–13, the laterals on joints 3, 4, and 6–12. The subdorsals of joints 3, 4, 5, 8, 11, 12, and 13 are large, the others small; the laterals of joints 3 and 4 are large, the others smaller, but not as small as the small subdorsals. Each horn bears three setæ, occasionally but two, slender, slightly clavate-tipped, smooth. The setæ of joint 2 are not elevated on horns. Skin smooth. Colorless, whitish, without markings. On hatching, the larvæ gathered in groups as they had been laid, and molted in two days. Length, 0.9 mm.

Stage II.—Head rounded, white, clypeus highly triangular, reaching about half-way to the vertex; eyes black; mandibles stout, brown-tipped. Elliptical, narrowed behind, dorsum nearly

level. Subdorsal horns of joints 3–5, 8, 11, 12 spherical, large, densely spined, those of joints 6, 7, 9, and 10 minute, with one tubercle and one spine only, and each approximated to the adjoining large horn; subdorsal horn of joint 13 small, with two or three spines. Lateral horns alike with three or four spines, except those of joints 3 and 4, which are larger and more rounded. Skin rather densely granular, smooth, without markings. The spines are large, with large tubercular bases and black tips. The detachable tip is nearly as long as the shaft of the spine and is not enlarged near the junction. These spines are presumably of the urticating type, as they have that structure, though they are probably too small to pierce the human skin, at least in this early stage. There is a row of fine hairs on the anterior edge of the hood (joint 2). Length, 0.9–1.6 mm.

Stage III.—Head elongate, higher than wide, whitish, the eyes in a round black spot, the mandible brown-tipped. Elongate elliptical, flattened, rounded squarish, narrowed behind more than in front, the dorsum flat. Subdorsal horns large, rounded, well spined, those of joints 6, 7, 9, and 10 represented by four or five spines on the skin, approximated to the neighboring horns. Lateral horns moderate, rounded, all elevated. Skin granular shagreened, the granules sharply conical over the dorsal region and separated by their own diameters or more, on the sides more flattened and irregular till along the subventral edge they form a pattern resembling alligator skin. All whitish green, no marks. The depressed spaces (1) are faintly indicated, paired. The cores of the large subdorsal horns are slightly whitish. The spines on the horns have the detachable tips relatively shorter than before, being not over one-third of the whole spine for the subdorsal horns. The lateral ones are less advanced, and have the structure of the subdorsals of the previous stage. Length, 1.6–1.8 mm. This is apparently the interpolated stage.

Stage IV.—Elongate, flattened, tapered behind, the ends truncate; the larva generally sits a little curved. Subdorsal horns rounded, large, subequal on joints 3, 4, 5, 8, 11, and 12, the pair on joint 13 small, not as yet produced. The short horns of joints 6, 7, 9, and 10 rounded, well spined, not so strongly approximated

to the neighboring horns as before. Lateral horns small, subequal. Skin densely granular shagreened. Depressed spaces (1) distinct, paired, a little whitish. Translucent greenish, a narrow white line along the subdorsal ridge the whole length. Horns slightly yellowish-tinted, with a trace of vinous shading at the ends of the body. Later there appear more decided traces of color. A yellowish-white band in the subdorsal ridge is broken into six slightly oblique broad bars under the long horns, elsewhere faint; there is a fine linear straight white dorsal line, broken, most distinct centrally, dividing the small white dots of depressed spaces (1). A slight purplish infiltration in the dorsal space between these marks. Length, 1.8–2.8 mm.

Stage V.—Elongate, flattened, tapered behind, the subdorsal horn of joint 13 about two-thirds as long as that of joint 12, the rest subequal, those of joints 6, 7, 9, and 10 very small. Lateral horns of joints 3 and 4 moderate, the rest small. Green, whitish along the sides; subdorsal horns yellow with a yellowish-white subdorsal band below them, narrowed in the spaces between to border the dorsal space, which is especially elliptically widened at the short horns of joints 6–7 and 9–10. A broken white dorsal line; depressed dots (1) greenish, impressed. A slight purple infiltration all along the dorsal space between the markings. Length, 2.8–4.1 mm.

Stage VI.—Horns of joint 13 distinctly elongated into tails, longer than the subdorsals of joint 12. Elliptical, the sides wide centrally and flattened, the dorsum long, straight, narrow. Horns moderate, the short subdorsals distinct, separate, about as large as the lateral horns, of which those of joints 3 and 4 are stouter, but hardly longer than the rest. Green, the subdorsal ridge broadly yellow, narrowed at the short horns, causing the dorsal space to expand there, apparently. A broken white dorsal line touching the white dots of depressed spaces (1). Dorsal space partly purple-filled. A broken yellow line beneath the lateral horns. Subdorsal horn of joint 3 slightly red-tipped. Spines black-tipped; skin densely frosty granular; the lateral depressed spaces show faintly as large, concolorous, kidney-shaped hollows. Later the markings become more pronounced. Green, the subdorsal horns of joint 3

red, those of joints 4 and 5 red-tipped, the rest green. Dorsal marking expanded in the intersegments 3–4, 4–5, 5–8, 8–10, and very slightly 10–11 and 11–12, the first and last green-filled, the rest dark purple, cut by the yellow dorsal line and the pale depressed spaces (1), broken at joint 8, broadly yellow-edged to the subdorsal horns. Horns with stinging spines, no caltropes. Skin sparsely clear granular. Length, 4.1–5.2 mm.

Stage VII.—As before. The subdorsal horns of joint 13 are long and tapered. The subdorsals of joints 3–5 are red, those of 12 and 13 slightly red-tipped; a greener line within subdorsal ridge. Length, 5.2–6.9 mm.

Stage VIII.—Subdorsal horn of joint 3 a little longer than those of joints 4 and 5, but all now very short, those of joints 6–11 still shorter, of 12 a little longer, of 13 long and tail-like. Side horns all very short. The level narrow dorsum is yellow and opaque, the dorsal band of purple widens into five subequal patches, nearly divided at joint 8, where the purple is replaced by red and is linear only. The last patch, on joints 11 and 12, is a little smaller; joint 13 dorsally green. Horns bright red. Sides all clear green; a broken white line along the lateral ridge and irregular white marks in the lateral depressed spaces. Skin granular; spines pale; no caltropes, except perhaps two or three on the basal anterior green spot of the horn of joint 13. Dorsal impressed dots (1) greenish with white rings. A dorsal white line narrowly cuts the purple. Length, 6.9–9.3 mm.

Stage IX.—Long, rather narrow, quadrate, a little tapering behind. Dorsum broad, flat, not arched and scarcely higher at joint 5, yet a little so. Subdorsal ridge indicated by change in direction. Sides perpendicular or nearly so, the lateral space broad, continuous with the subventral space which is infolded in the middle. Subdorsal horns distinct, short, those of joints 3, 4, 5, and 12 moderate, those of joint 13 long, nearly three times as long as the ones on joint 12, the rest short, those of joints 8 and 11 a little larger than the others. Side horns short, sessile, wider than long, those of joints 3 and 4 a little longer than those of 6–12. Caltrope patches on the horns of joints 6–12 and on the base of the subdorsal horn of joint 13, large on joints 12 and 13, then progressively smaller

till the horns of joints 6 and 7 have only a few or no caltropes. Skin finely clear granular except on the horns. No end spines. Dorsum yellow- or red-shaded, a purple band with white glandular dots and central dorsal line much as in *spinuloides*, but of different shape. It widens between joints 3 and 4, 4 and 5, then moderately widens on joints 6 and 7, narrows to a slight bordering of the white dorsal line over joint 8, widens behind the horns on 9 and 10, moderately widens between joints 11 and 12 and ends, joint 13 being green above. A bright red, diffuse, subdorsal band; all the subdorsal horns red. Below, a yellow stripe, narrowly red-edged, waved. Sides green, a row of yellow dashes along the lateral horns, green-edged above; yellow rings on spaces (4). A white line along the subventral edge. Stinging spines short, not numerous. Depressed spaces (1) and (2) represented by white dots, (1) paired and on joints 3–4 and 4–5 also double; depressed space (4) reniform, distinct; slight hollows subventrally; spiracle of joint 5 moved up out of line. Length, 9.3–13 mm.

Cocoon.—Nearly spherical, hard, brown, one end opening like a circular lid on emergence, though there is no sign externally of the crevice of the lid. Spun with a slight veil against a leaf. Diameter, 5 mm; length, 7 mm.

Pupa.—With the characters of the family. Delicate, thin-skinned with the members free. It emerges nearly out of the cocoon when the adult issues.

Food plants.—The red oak is the preferred plant, though the larvæ will feed on almost any smooth-leaved tree. I found one on maple at Tryon, the others mostly on oak. In confinement my larvæ fed on yellow birch, which they seemed to prefer to soft maple.

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PLATE 2.

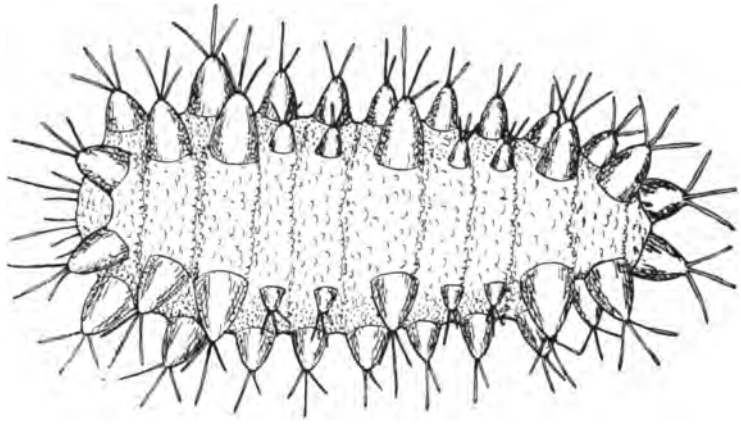

FIG. 1.

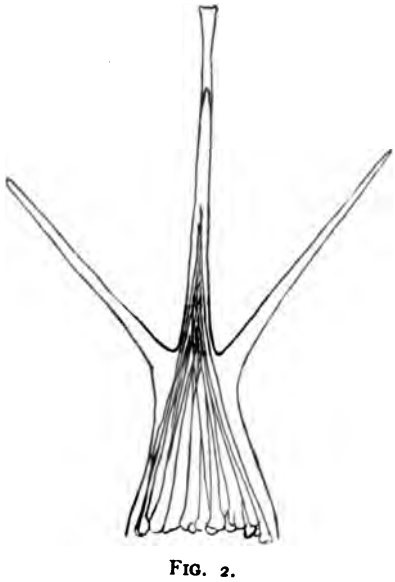

FIG. 2.

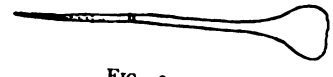

FIG. 3.

FIG. 6.

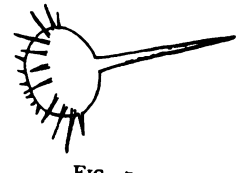

FIG. 7.

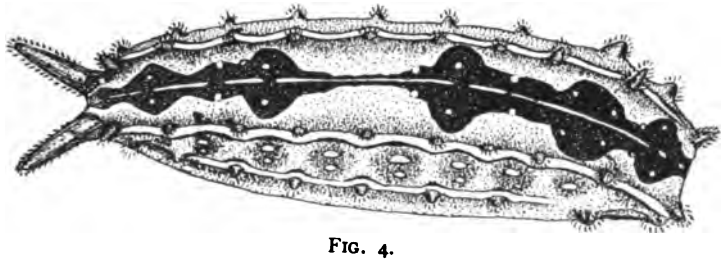

FIG. 4.

FIG. 5.

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EXPLANATION OF PLATE 2.

FIG. 1.—The larva in Stage I, dorsal view.

FIG. 2.—One of the subdorsal horns of Stage I, the spines of the next stage appearing by transparency. The larva was about to molt.

FIG. 3.—A spine of Stage II.

FIG. 4.—The mature larva in position of feeding.

FIG. 5.—A seta of the last stage.

FIG. 6.—An urticating spine of the last stage.

FIG. 7.—A caltrope spine of the last stage.

EXPERIMENTAL METHODS AS APPLIED TO WATER- AND SEWAGE-WORKS FOR LARGE COMMUNITIES.*

GEORGE W. FULLER.

THAT progress means advance in knowledge and the gradual transition of information from the unknown to the known is of course a truism. The manner by which progress is made in different lines of work varies widely. The experimental method as applied to the teaching of science in educational institutions has been of great benefit, and the application in a more systematic manner than formerly of the so-called "cut and try" method of the early inventors has resulted in more substantial progress in many mechanical and industrial lines. By the man of affairs this advance in knowledge is referred to as additional experience. It is not the purpose of this paper to attempt an analysis of the manner by which knowledge in general is advanced, but to refer somewhat briefly as a matter of record to the way in which there have been solved various sanitary problems which a quarter of a century ago, for financial or other reasons, seemed out of question.

It is fitting on this occasion that this topic should be touched upon, owing to the influence exerted upon this line of work by the two institutions with which Professor Sedgwick has been most actively connected in recent years, viz., the Massachusetts State Board of Health and the Massachusetts Institute of Technology. The former has for years been the foremost among the state boards of health of America in leading local authorities to improve their water- and sewage-works in an efficient and economical manner. Its influence has extended, not only to other states, but also to numerous places throughout the civilized world. The Massachusetts Institute of Technology has exerted a less direct influence; but, as a training school for many who have taken a prominent part in improving water- and sewage-works, this institution and a number of its

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teaching staff have achieved results which will be more fully appreciated as the years go by.

While the experimental method so successfully applied in the student laboratory may in its way call for just as conscientious and diligent effort as when applied to projects involving immense sums of money, yet the responsibility associated with the latter is far greater. It is necessary, in carrying out successfully these large practical problems, not only to draw correct conclusions from full representative premises of a complicated nature, but also to adjust the project to a reasonable business basis, to make it fairly well understood by non-technical officials and citizens, and to defend it from the obstructionists who, for political or selfish reasons, cross the pathway of nearly every large public enterprise. In meeting these requirements there has been called forth a series of efforts which are of great significance to the public from the sanitary and financial standpoints, and which form notable achievements in the field of applied science.

BENEFITS OF IMPROVED SANITARY WORKS.

Improved water- and sewage-works of course do not explain by any means the entire improvement which for the past quarter of a century has been so characteristic of the sanitary conditions of a majority of the civilized communities of the world. But, illustrative of the scope of such improvements under the guidance of wise sanitary authorities, it is of interest to point out the markedly decreased death-rates in Massachusetts from water-borne diseases, of which typhoid fever is the typical, but not the only one.

DEATH RATES PER 100,000 POPULATION FROM TYPHOID FEVER IN THE STATE OF MASSACHUSETTS BY FIVE-YEAR PERIODS FROM 1881 TO DATE.

Period	Rate	Period	Rate
1881–1885	41	1896–1900	26
1886–1890	46	1901–1904	19
1891–1895	34		

What is true of Massachusetts is in a general way true of many sections of both America and Europe where the population has become quite dense, and demands for water- and sewage-works of satisfactory character have pressed forward for attention in recent

years. No attempt will be made here to show the great sanitary benefit derived from other factors than improved water supply and sewerage, as this matter is clearly set forth in standard works upon sanitation and official reports from various quarters of health authorities who deal with the accomplishments of the modern health officers.

It is sufficient here to present clearly to the reader the thought that modern sanitary science has greatly increased the comfort and safety of living. In various cities the reductions in death-rates have been far greater than as given above for the state of Massachusetts. This is especially true in cities where badly polluted water supplies have been replaced by improved supplies. Numerous instances of this sort are on record where annual typhoid fever deaths of 50 to 100 per 100,000 have been reduced to about 20. These cities include not only those now receiving upland waters from unpolluted sources, and ground water, but also those having filtered water from earlier but polluted sources; for example, Lawrence, Mass., Albany, N. Y., York, Pa., and Lorain, Ohio. Intestinal diseases other than typhoid fever have been so reduced as to lower the general death-rate materially. For low-lying communities like New Orleans modern drainage has lessened notably the general death-rate, and sewerage brings safety as well as comfort to communities, among other ways by eliminating privy vaults and the likelihood of disease transmission by flies, etc.

As to financial considerations, it is difficult to present the full significance of this feature to the general reader without statistics which would be out of place in a short general article like this. Usually water-purification projects proper cost about $3 to $5 per inhabitant served; but pumping, force-mains, conduits, reservoirs, and other associated appurtenances sometimes increase the investment to $15 to $25 per capita. Sewage purification frequently is more expensive than water purification. Upon capitalizing the operating expenses, it is found that modern sanitary works, while involving only small costs for the individual, reach sums of millions and tens of millions of dollars for our large cities. The solution of these problems has brought many added duties to sanitary authorities, to professional men who assume the responsibility for con-

structing and operating the required works, and to the educational institutions which give technical training to young men desirous of entering this field of work.

NEW CONDITIONS WHICH HAVE BEEN ENCOUNTERED.

Twenty-five years ago the large cities of America were, of course, provided with public water supplies, and many of them had progressed considerably in adopting sewerage systems, although these latter now appear to have been more or less crude. Sewage-purification plants and water-purification plants, with perhaps half a dozen small and scattered exceptions, were practically unknown. A large proportion of the water supplies, especially in the Central West, were seriously objectionable in the excessive quantities of mud which they carried, and so different in their nature from the comparatively simple filtration projects of Europe that engineers naturally hesitated for financial reasons to attempt their construction, to say nothing of the question whether they would be able to give satisfactory service. The bacterial and hygienic aspects of these problems, which are now recognized to be of so much importance, were almost unknown. In fact, the germ theory of disease had not risen to general acceptance.

The medical man interested in public health knew in a general theoretical way what he wanted, but he was ordinarily unable to state his requirements in a manner understood by the engineer or by the taxpayer. Engineers were able to build any reasonable works, but were unable to learn in terms of the constructor what was required with sufficient definiteness to allow them to make even preliminary sketches and estimates of cost. The chemist and bacteriologist occupying an intermediate position produced with ill-suited methods analytical data full of mystery for everybody.

Misunderstanding continued until men interested in various lines of applied sanitary science co-operated in a manner to make themselves mutually understood. The successful movement to this end, at least in the United States, had its inception chiefly in Massachusetts some 20 years ago. It has resulted during the past dozen years in some notably well-balanced designs for American water- and sewage-works, which have demonstrated their sound

merit in practice. In this article it is the endeavor to outline the development of this aspect of experimental methods.

EXPERIMENTAL METHODS IN MASSACHUSETTS.

The experimental methods which have been put in practice in America so much in recent years may be defined as the bringing-together of reliable preliminary data from the engineering, chemical, bacterial, and hygienic standpoints, in order that efficient sanitary works may be built for a wide range of local conditions within the limits of reasonable cost; and if data are inadequate for fair assumptions, then the procuring of the needed data by practical tests on a small scale.

It is the Massachusetts State Board of Health to which credit is principally due for developing this method to serve as a guide for such works. In 1886, when the present board was organized, one of its first steps was to establish the Lawrence Experiment Station, whereby data were to be secured to show the best means available under various local conditions for purifying water and sewage. The legislature enacted that this board should serve as a sanitary tribunal, before which the local authorities should place their projects for water- and sewage-works, and whose approval was requisite before the state authorities granted the local authorities permission to issue bonds for their construction. This experiment station has been in continuous service since the autumn of 1887, and has attained a high reputation among various workers in the field of sanitary science throughout the world, in addition to fulfilling its main purpose of aiding the citizens of Massachusetts in economically improving their public works, whereby to a material degree the health and comfort of the people of the state have been enhanced. These results are so well known that it is needless here to go into detail.

The classical investigations at Lawrence, as set forth in the annual reports for the past 15 years, have undoubtedly done more than any other series of investigations in the world to place the science of purifying water and sewage on a sound practical basis. It is true that earlier workers abroad had previously taken important steps along some of these lines, and that sand filtration of water had for

many years been in use. But they did not secure comparable parallel data from the engineering, chemical, and bacterial standpoints with anything like the completeness obtained at Lawrence, whereby the laws governing successful practice could be broadly stated for a wide range of conditions.

Not only has the Massachusetts State Board of Health availed itself of a testing department, but with other departments it has placed itself in a position to utilize such data advantageously. This has been done by an analytical department procuring data at frequent intervals as to the character of various water supplies, rivers, effluents, etc., and, more especially, by a well-trained engineering corps which applies the various data to the needs of each problem coming to the attention of the board.

That the Massachusetts State Board of Health handles well the work coming within its jurisdiction is conceded by all in a position to know of it intimately. It is true that the board is criticised for not devoting itself more enthusiastically to studies of methods finding favor elsewhere, but this criticism has little to support it. The board properly confines itself to the solution of problems within the state, and of course does not consider it necessary to do more than keep generally familiar with other methods, no matter how suitable they may be for work elsewhere.

EXPERIMENTAL METHODS ELSEWHERE IN AMERICA.

In water purification the Massachusetts problems are, generally speaking, much easier and simpler than those of the Central West and South, where enormous quantities of silt and clay complicate the necessary works for treating the water, and add materially to the cost as regards both construction and operation. In a manner similar to the procedure at Lawrence, these problems were worked out at Louisville, Pittsburg, Cincinnati, and New Orleans. At numerous other places the experimental method has been used in adapting more strictly the design of works to local conditions, especially in the preliminary treatment of turbid waters (Philadelphia and Harrisburg), the removal of color from surface waters (Providence), of iron from ground waters (West Superior), the softening of hard waters (Columbus), and the removal of tastes

and odors (Reading and Springfield). These problems have all been carefully studied in small test devices for securing data necessary for advantageous design and operation.

Sewage purification has also been studied under various local conditions at several places, especially at Worcester, Mass.; Pawtucket, R. I.; Berlin, Ont.; the Institute of Technology, Boston; Columbus, Ohio; and Waterbury, Conn. In most cases the sewage studies have arisen because of inability or great expense in applying the well-known Massachusetts method of intermittent filtration through sand, or because of peculiarities of the local sewage.

A partial list of the more prominent investigations as to purifying water and sewage, with dates and approximate costs, is as follows:

LIST OF SPECIAL INVESTIGATIONS ON WATER AND SEWAGE PURIFICATION.

Place	Date	Work	Approximate Cost
Lawrence, Mass.	1887 to date	Water and sewage	$175,000
Providence, R. I.	1893–94	Water	5,000
Louisville, Ky.	1895–97	"	47,395
Reading, Pa.	1897	"	1,500
Pittsburgh, Pa.	1897–98	"	36,286
Cincinnati, Ohio	1898–99	"	41,588
West Superior, Wis.	1898–99	"	2,000
Washington, D. C.	1899–1900	"	8,000
Richmond, Va.	1900	"	2,000
New Orleans, La.	1900–1	"	23,606
Worcester, Mass.	1900 to date	Sewage	37,000
Philadelphia, Pa.	1900–5	Water	172,000
Springfield, Mass.	1901–3	"	18,000
Harrisburg, Pa.	1903–4	"	25,000
Massachusetts Institute of Technology, Boston	1903 to date	Sewage	20,000
Columbus, Ohio	1904–5	Sewage and water	44,004
Waterbury, Conn.	1905 to date	Sewage	10,000
Total			668,379

It is not pretended that the above list is complete. In fact, there are other tests which, while small and of short duration, have had much to do with professional opinion. Perhaps the most important were demonstrations at Louisville and St. Louis, many years ago, that plain sand filtration was incapable of treating the muddy Ohio and Mississippi River waters after plain sedimentation in large basins.

The benefit derived from the experience of the owners of proprietary devices cannot be overlooked—especially in regard to various appliances of mechanical filters which occasioned the expenditure of much money before being brought to their present state of development. At Louisville alone the five competing filter companies

spent more than $50,000. More recently their devices, when tested, have been purchased at the beginning.

While this paper is devoted essentially to methods of purifying water and sewage by works partly or wholly of artificial construction, mention should be made of the important advances in the allied field of water supply from storage reservoirs, and the disposal of sewage by dilution. Among the more prominent investigations of this kind should be stated those field surveys and laboratory studies made in connection with the Chicago Drainage Canal, the additional water supply of New York City, the improvement of the Charles, Mystic, and Neponset Rivers in Massachusetts, and foreshore pollution along the Massachusetts coast. Several hundred thousands of dollars have been expended on these investigations.

OBJECT AND ADVANTAGES OF EXPERIMENTAL METHODS.

The purposes of applying experimental methods to problems of water and sewage purification are chiefly threefold, as follows:

1. To provide data for the official and technical authorities, to enable them to adapt new works most advantageously to the local conditions, and to indicate dimensions and other physical conditions permitting contract plans to be prepared and the cost of construction to be approximately estimated.

2. To educate the non-technical public, who as citizens and taxpayers are interested in public works.

3. To provide data so that the officials can operate effectively the works after they are completed, and forecast the approximate cost of operation.

Technical data.—In regard to the first object accomplished, that of enabling city officials and their technical advisers to design economically works of a suitable character, it goes without saying that this has been of the greatest importance, and is a strong factor in explaining the rapid strides in successful sanitary works accomplished during the past few years. It has frequently been the advice of technical men, in dealing with problems which differ from those successfully solved elsewhere, to make tests for a year or so at a cost approximating the interest for one year for the works contemplated. In this way the cost of errors and unbalanced designs

has been largely minimized, and the efficiency has been increased. In the field of water and sewage purification the information and experience now available are sufficient in a majority of cases to enable these problems to be advantageously handled by experienced workers along these lines. There are some problems, however, that still can be advantageously treated by the experimental method. They refer especially to sewage problems in which trade wastes are involved, and to water problems where the composition of the water is quite unusual in some particulars on frequent occasions. From the technical standpoint, however, the field of water and sewage purification, broadly speaking, has passed beyond the experimental stage, and the advances, both as to efficiency and economy, are largely to be gained, not from experimental plants, but by the careful and more systematic operation of works in practice. Such studies will, of course, lead to improvements which can be taken advantage of in the construction of new works, and will gradually bring to a higher plane of excellence the art of water and sewage purification on its present scientific basis.

Educational aspect.—It is frequently said that communities progress in proportion to the advance in knowledge of the average citizen, or to the mean knowledge of the community as a whole. There is a good deal in this, and it brings forcibly to mind the necessity of educating the public as to what improved sanitary conditions really mean, and of letting them ascertain for themselves what can be accomplished along these lines in the field of applied science. Non-technical people have a natural aversion to the word "experiment," notwithstanding the aid derived from devices which not improperly may be termed experimental. While the term "experiment station" from its use at Lawrence and a few other places seems to have a firm footing in some localities, it is gradually giving place to the term "testing station." This is a much preferable expression in many ways, as it disarms the criticism of many who seem to think that these investigations are conducted in a "hit or miss" manner, much after the fashion of the early inventors. This is not so, as experimental methods, as now ordinarily applied to water- and sewage-works, are aimed at testing procedures found successful elsewhere, but which may require adaptation to local

conditions in regard to some details. Their magnitude, while relatively small for reasons of economy, is still much greater than that which seems to be taken for granted by numerous citizens, who associate the word "experiment" with a test tube, or with a mechanical device which is so imperfect that no one dares to build it on a large scale without further experiments. The methods of purifying water and sewage have now advanced to a degree where the phrase "testing station" in new projects will unquestionably displace "experiment station;" and the testing of these processes where unusual conditions are expected will assume a dignity comparable with that of the regular departments which systematically test cement, steel, and other materials used for building purposes. In fact, it is interesting to note that the laboratories at many testing stations have been utilized regularly for testing construction materials.

Where water- and sewage-purification projects involve hundreds of thousands of dollars or more for construction costs, the so-called experimental methods, as applied in accordance with the foregoing statements, have given wonderful courage in many places to officials who otherwise would very naturally have been in a hesitating frame of mind, and inclined more to listen to the "doubting Thomases" who in all communities, for selfish or other reasons, appear as opponents and obstructionists to modern sanitary works. Even if the technical advisers of the projects were not assisted by such data, it is quite likely that the testing station for many projects would indirectly in this way do far more good than the cost involved, in saving lives and in hastening the day when communities will meet their problems in accordance with the best information available.

In speaking of the educational benefit derived from applying experimental methods to water- and sewage-works, the technical men, especially those in charge of the tests, have an important duty to perform in teaching non-technical officials, and various citizens who are interested in the work, the fundamental principles of the process involved, and in assisting them in ascertaining what practical works would mean, both hygienically and financially. Along this general line the Institute of Technology has played an important rôle, largely through having had for many years on its teaching staff a man who to an extraordinary degree possesses the faculty

of getting fundamental truths of sanitary science before his hearers in such an attractive manner that they never forget them. It is the belief of the writer that the work accomplished by Professor Sedgwick along this line is unequaled by that of any other man in this country, either in educational or other lines, and that this fact in a few years will be far more widely realized than at present, when his younger pupils throughout the country reach an age where their work will be felt in the communities in which they live. This influence is already to be found in many unexpected places, and forms a wonderful tribute to the success accomplished by Professor Sedgwick in one of his many lines of usefulness.

Operation of works.—After water-and sewage-purification works are constructed, it is imperative that they shall be operated in an intelligent and efficient manner. The benefit of this has long been demonstrated in Europe, and the absence of such supervision in many places in America shows the folly of careless and indifferent management. No matter how well water- and sewage-purification works may be designed and built, there is no engineer who can give assurance that the results accomplished will be satisfactory unless the works are well managed. Not only must the works produce a result which is satisfactory from a scientific standpoint, but their behavior should be put before the citizens in a way that will inspire confidence. When fair-minded citizens as a mass continue to lack confidence in works of this type, the latter cannot be called an unqualified success, no matter how fully scientific facts may show their adequacy.

The Massachusetts Institute of Technology instituted the plan of especially training young men along technical lines, so that they might become competent to serve as superintendents for water and sewage-purification works. In this pioneer work they are entitled to great credit, and their example is already being followed by similar institutions elsewhere. This is an important field of technical education, as a majority of such technically educated men in the future will be connected with the management, rather than with the construction, of works of this type.

In passing, it may not be amiss to say that the technical managers of works of the type under consideration must have other quali-

fications than those of a scientific nature. They must be able to maintain amicable relations with executive superiors, to manage laborers, to keep records in a manner fairly comparable with the high degree to which the art of bookkeeping in large business houses has advanced, to prepare reports containing essential features in explicit but terse terms, and to make plain to non-technical men in both public and private capacity the more essential features of their own position and of the data by which their efforts show what is being accomplished. This type of specialists will naturally develop in efficiency as their responsibilities increase; but there is still much work for the technical schools to do in preparing young men more adequately for these duties.

Tentative installations.—As distinguished from the testing stations built solely for the purpose of tests, there is, of course, one other method of a somewhat experimental nature by which local data are used in determining whether large works are most advantageously constructed. I refer to the plan of constructing works gradually, or tentatively, and of using data from the operation of the first portion of the installation to serve as a guide in arranging the details of the portions subsequently to be built, and also in deciding upon the magnitude of the works sufficient for a given capacity or to serve for a given term of years. This is the style of works, from the experimental point of view, which frequently obtains in Europe, and which will obtain in some places in this country. As yet there has not been a wide application in America of such data obtained on a large practical scale, although, of course, they are availed of more or less in all works where extensions are required. This condition has been reached at several sewage-works in New England, and the results of experiences in the field have been summarized by the Massachusetts State Board of Health. It is gratifying to state that practical results are in general conformity with the principles of water and sewage purification as developed by tests on a small scale.

EXPERIMENTAL METHODS IN EUROPE.

In Europe the water-purification problems do not cover nearly so wide or difficult a range of natural conditions as those met in America. Filtration has in recent years not received as much attention experi-

mentally as has been the case with sewage-works. In earlier years however, experimental methods had much to do with the development of water filters abroad. It is not to be forgotten, furthermore, that in Germany much good work during the past dozen years has been done in developing the most practical methods for removing iron from ground waters. At present the most interesting feature of water-purification developments in Europe refers to the preliminary treatment for some of the river waters which are fairly turbid during freshets, and to efforts to sterilize water economically by ozone. The most notable instance of the former is at Suresnes, near Paris, where the Seine water below the metropolis is subjected to filtration six times, the first filters being of coarse gravel to effect clarification.

In England, which is the home of modern sanitary engineering, sewage-purification works have received more attention than in any other country. The density of population in England and the relatively small size of its rivers have, of course, forced this condition at an earlier date than is generally true of other countries. While for some years the English have not contributed much on the subject of water filtration, their experience in the field of sewage purification far exceeds that of any other country. Experimental methods in one form or another have played an important part for half a century, beginning with efforts to utilize the manurial value of sewage. This is largely owing to the differences in various local conditions, especially topography, geology, and the composition of the sewage as influenced by trade wastes. Not only have the English conducted test filters and other processes of purification on a small scale, but they have also gathered many data of great value by the operation of their works in practice along lines which enable current experiences to be utilized in developing future works.

These data have been so universally obtained in conjunction with the operation of existing works in practice that it is very difficult to ascertain even roughly what their cost has been. The staff regularly engaged in operating the main works has secured the technical data, so that the expense has been confined to building the test devices, relatively small in size, and to a little extra labor for operation. The large mass of valuable testimony published in numerous

municipal reports and by the Royal Commission on Sewage Disposal shows what a fund of knowledge has been accumulated at London, Salford, Sutton, Exeter, Burnley, Accrington, Huddersfield, Leicester, Birmingham, Bradford, Devizes, Hanley, and other cities, and which for most places has been obtained with almost no special fund devoted to testing purposes, comparatively speaking.

At Leeds the unusually thorough sewage tests made during the past eight years received appropriations of about $150,000, some two-thirds of which has been actually devoted to that purpose. Manchester has also expended quite large sums for experimental purposes, although, for the reasons above stated, the expenditures were by no means commensurate with the information obtained. The Royal Commission on Sewage Disposal in England is understood to have an appropriation of about $55,000 for the expenses of its own staff and the traveling expenses of the numerous witnesses who have appeared before it. There are also special river boards and county councils, with excellent technical staffs, which gather many valuable data.

In France sewage purification has been the subject of experimental study, beginning with the labors of Mille in 1868 at Gennevilliers. These tests resulted in the establishment of the present sewage farms of Paris. Within the past few years the biological methods of purification have received attention both from the city of Paris and from the Department of Agriculture. The latter has a general supervising control over water and sewage matters outside of Paris, and is devoting an appropriation of about $60,000 to such investigations. Thus far these studies have been made by Professor Calmette at Lille, as set forth in his interesting progress report of last autumn.

In Belgium the government is paying particular attention experimentally to the treatment of trade wastes at a special station devoted to that purpose at Verviers.

The government of Holland established, in 1904, a sewage-testing station at Tilburg, the cost of which to date is approximately $15,000. No reports have yet been published. Several ozone plants have been tested in Holland, and the city of Rotterdam is now arranging to test a mechanical filter on the local river-water supply.

In Germany numerous experiments have been made upon the sedimentation of sewage for purposes of clarification, and the so-called biological methods have been studied for some years, beginning in 1895, when a testing station was established by Professor Dunbar at Hamburg, which station is still in operation. In 1901 the Prussian government established a permanent organization for testing water- and sewage-purification methods. This "institute" has gathered together and published the more important data as to experiences in other countries, has conducted several important sewage-testing stations in the suburbs of Berlin, and has collated many useful data as to the sanitary works of the cities of Prussia and neighboring territory. This department has an annual appropriation of about $30,000 for testing, inspecting, analytical, and clerical purposes. The sum devoted to testing purposes varies, but is materially supplemented by the arrangement of conducting investigations for various local authorities, the expense for which is borne in part by the community benefited. The department also established the custom of officially examining proprietary devices, largely at the expense of the owners in cases where the devices seem to possess sufficient merit. In this way a mechanical filter of the Jewell type was recently tested at the Müggelsee plant of the Berlin water-works. The same filter is now being tested on the colored water supply of Königsberg.

The relative amounts of suspended matters deposited from sewage at different velocities have been studied carefully under varying local conditions at Frankfurt, Cassel, Hannover, and Cologne, as shown by the data published in municipal reports and the technical press. In these cities, as in England, it is difficult to ascertain the cost of the tests, because so much of the work was done by the regular staff of the technical authorities of the cities. The scope of the tests has probably been greatest at Frankfurt, including means for most easily removing sludge, its partial drying by centrifugalization, and its ultimate disposal by incineration after mixing with the city refuse. About $60,000 has been spent at Frankfurt on these and other sewage tests, including filtration, within the past dozen years or more.

Professor Dunbar's activities in the field of sewage purification

have by no means been confined to Hamburg. His publications show that he has advised the authorities at Mühlhausen, Stuttgart, Beuthen, Unna, Leipzig, and other places. In nearly every instance he has taken advantage of experimental data to ascertain local conditions. Leipzig and Chemnitz in Saxony are now conducting sewage tests, the appropriations for which are about $17,000 in each case, with the engineering and analytical data secured by men regularly employed by the city.

This brief record of experimental methods as applied to water and sewage purification can hardly be brought to a close without reference to trade wastes. This feature in aggravated cases complicates the design of sewage-works and adds materially to the costs of operation. Various industries require special consideration, as shown by the efforts of the river boards to minimize the effect of trade wastes in the streams of Lancashire and Yorkshire, England. The removal of fats has perhaps received the most attention along this line—especially in Berlin, Cassel, and Chemnitz in Germany, Verviers in Belgium, Roubaix and Grimonpont in France, and Bradford, Manchester, and Oldham in England. Numerous mill-owners also recover grease from their waste water. The extent of some of these investigations is indicated by the fact that at Cassel a private company is said to have spent considerably more than $100,000 in unsuccessfully endeavoring to fulfil a contract for extracting fats from the city sewage. The only large place where the entire city sewage is regularly treated for grease extraction is at Bradford, England.

THE FUTILITY OF A SANITARY WATER ANALYSIS AS A TEST OF POTABILITY.*

MARSHALL O. LEIGHTON.

WHOSOEVER expresses doubts concerning generally accepted ideas must be prepared to see his statements misinterpreted and their application carried far beyond the point at which they were aimed, even to the absurd and grotesque. He must not expect that his observations and deductions will be confined to the limits prescribed, even though he resorts to every safeguard that his mother-tongue affords. More attention is paid to the devious paths along which his statements may lead by implication than to the single trail that he has defined by precise guide-posts. Finally, such a person must sustain confrontation by that splendid, indispensable, and all-saving power known as conservatism. Therefore the author of this paper hoists a flag of truce while he makes his preliminary declaration, in the hope that the highest possible proportion of those interested may not mistake his line of march.

1. *All contentions concerning the futility of sanitary analyses are applied strictly to waters.* Sewages, fresh and stale, and sewage effluents are expressly eliminated from consideration, except in certain cases where they will be taken to illustrate the fact that they may occasionally be accepted as unpolluted water, according to standard methods of interpretation.

2. *It is not contended that all sanitary water analyses are futile* irrespective of the conditions under which they are made and interpreted. In consistent studies of nitrogen, as such, and the changes which take place in its form, such analyses are important.

3. *It is admitted that in certain limited areas of the United States sanitary water analyses afford information by which animal pollution may occasionally be detected.*

4. *The facts comprised in the foregoing admission have been a stumbling-block* to chemists working with waters outside of those limited areas.

* Received for publication, March 30, 1906.

5. *It is contended that the sanitary analysis offers nothing by which one may positively distinguish between a dangerous and a wholesome water.*

6. *The composition ratios that many good men cherish may be applied indiscriminately to wholesome waters and dilute sewages.*

7. *The conventional method of seeking for evidences of pollution by sanitary analyses, or of accepting or rejecting a water upon such evidence, is in its broad and essential features quite misleading, too frequently dishonest, and in some cases absurd.*

8. *The dangerous pollution of surface waters can be discovered more readily, and at far less risk and expense, than by sanitary analysis.*

9. The term "sanitary analysis" as used in this discussion does not include tests for specific organisms.

Standards of interpretation by which a water may be designated as "good" are faithfully met by many waters undeniably bad; conversely the characteristics of a water interpreted as "bad" are presented by many the wholesomeness of which cannot be questioned.

There is in the presidential address of Professor Leonard P. Kinnicutt, delivered before Section C of the American Association for the Advancement of Science, at New Orleans, La., in December, 1905, a concrete statement of intrepretation standards. This statement will be used as a basis for the comparisons which follow in this discussion. Such a selection is made, decidedly not for the purpose of controverting the statements or discrediting the position of this distinguished authority, but rather because it is the most admirable résumé that has recently emanated from a highly respected and competent source. The following statements are quoted:

(A) In fresh sewage the amount of nitrogen as free ammonia is from three to four times that of the nitrogen in the albuminoid ammonia, and in sewage effluents from 20 to 30 times, while in peaty water, or water containing an infusion of leaves, the nitrogen in the albuminoid ammonia is from 10 to 20 times the nitrogen in free ammonia. Hence, when a surface water, not including rain or snow water, gives a greater amount of nitrogen as free ammonia than it does as albuminoid ammonia, the indications are that the water has certainly been polluted by sewage, and that the source of the organic matter is of animal origin. With a large amount of nitrogen as albuminoid ammonia (over 0.25 milligram per liter) a ratio of nitrogen of the free ammonia to the nitrogen of the albuminoid ammonia of less than 1 to 5 is suspicious.

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(B) Consequently, while a low ratio as 1 to 5 between the nitrogen of the free ammonia and the nitrogen of the albuminoid ammonia indicates pollution, the reverse cannot be said to be a strong indication that the water is a normal water.

(C) A colorless water containing that amount of nitrogenous matter represented by 0.25 milligram of nitrogen as albuminoid ammonia per liter is looked upon with suspicion.

(D) Free ammonia always indicates organic matter in the process of decomposition. In unpolluted surface waters it is rarely high, being removed almost as fast as formed by vegetable and animal organisms in the water, and an amount of nitrogen as free ammonia above 0.05 milligram per liter is unusual, and, if it does occur, the water cannot be considered as an unpolluted water unless that fact is clearly established by other data.

Attention is then called to seasonal variations and the increase in free ammonia during the autumn in northern countries.

(E) Concerning nitrogen as nitrites:

More than 0.002 milligram per liter is an unfavorable indication.

(F) Concerning nitrogen as nitrates:

It is never present in any large amount, seldom exceeding 0.1 milligram per liter. Higher amounts than this, being unusual, must be looked upon with suspicion.

Professor Kinnicutt then explains that the above interpretations refer to reservoir, pond, and lake waters, but that in river waters high nitrogen as free ammonia, as albuminoid ammonia, and as nitrites characteristic of recent pollution in ponds and reservoirs may be due to the decomposition of algæ life, which was stimulated by the entrance of sewage in the upper stretches of the river.

Accepting the above as an authoritative basis of interpretation —and it is the one which closely corresponds to that which the writer has found in very general use—let us interpret a few analyses. Reference will be made by letter to the foregoing quotations, so that the basis of each interpretation may be clearly defined.

SERIES "A."
PARTS PER MILLION.

No.	Date	Turbidity	Color	Odor	Nitrogen as—				
					Albuminoid Ammonia	Free Ammonia	Nitrites	Nitrates	Chlorine
1	July 11, 1900	0	0	0	0.026	0.028	0	0	1.2
2	July 20, 1899	Cons.	1.5	1m	0.330	0.246	0	0	1.2
3	Sept. 14, 1900	Sl.	2.1	1m	0.114	0.164	0	0	1.2

There are presented in the above series three analyses of pond waters. All are practically colorless, and Nos. 2 and 3 have a

slightly moldy odor. (A) In Nos. 1 and 3 the nitrogen as free ammonia is greater than that as albuminoid ammonia. (D) Nos. 2 and 3 contain very much greater amounts of free ammonia than 0.05 part per million. (A, second part) No. 3 contains over 0.25 part of albuminoid ammonia per million, and the free-albuminoid ratio is 1 to 1.3. No nitrites or nitrates appear in any of the samples. According to the above standards of interpretation, all three of these waters contain recent organic pollution of a very unstable nature or, in other words, sewage pollution; the nitrification is proceeding very rapidly, and the assimilation of nitrites and nitrates is accomplished as rapidly as they are formed, by an abundance of organisms. In point of fact, these are normal waters from two storage ponds in Pennsylvania, in the drainage areas of which there are no habitations. It would be difficult to specify conditions that would more closely approach the ideal for upland conserved supply than existed at these two places at the time these samples were taken. No. 1 is from Pine Run Reservoir, and Nos. 2 and 3 from Mill Creek Reservoir, both in Luzerne County, Pennsylvania.

SERIES "B."

PARTS PER MILLION.

No.	Turbidity	Color	Odor	Nitrogen as—				Chlorine	Total Residue	Loss on Ignition
				Albuminoid Ammonia	Free Ammonia	Nitrites	Nitrates			
1	Dist.	13	2a	0.120	0.006	0.000	0.170	0.9	69.5	19.50
2	"	15	1a	0.204	0.016	0.000	0.190	0.9	72.0	20.00
3	"	8	2a	0.106	0.002	0.000	0.160	0.9	66.5	16.00
4	"	8	1a	0.142	0.026	0.000	0.160	0.9	66.5	13.50
5	"	13	1a	0.130	0.012	0.000	0.160	0.9	69.0	20.00

Series "B" contains analyses of samples taken from a large lake in September, 1904. Each sample was distinctly turbid, of low color, and revealed an aromatic odor. (C) The nitrogen as albuminoid ammonia is in every case less than 0.25 milligram per liter. (D) The nitrogen as free ammonia is in all cases far less than 0.05 milligram per liter. (A) The free-albuminoid ratio varies from 1–5.3 up to 1–5.5. (E) There are no nitrites. (F) The nitrates run somewhat higher than the standard set.

Several of the above samples have all the characteristics of an

infusion of leaves (A) except color. There is nothing in the analyses presented, except the unimportant excess of nitrates, that does not surpass on the acceptable side the strictest of the interpretation standards above quoted. The samples were collected almost simultaneously on September 22, 1904, from Lake Champlain, within the inclosed area lying between the docks at Burlington, Vt., and the harbor breakwater. Sample No. 2 was taken about 1,000 feet away from the outlet of the main trunk sewer of the city. The remainder were taken at points less than 500 feet away fom said outlet, No. 5 being collected about 20 feet from the sewer's mouth. Ten years previous to the collection of these samples the city of Burlington was obliged to abandon a water intake situated at a much more favorable point than those at which any of the above samples were taken. The reason for the change was the high rate of intestinal disease morbidity in the city, which was markedly decreased afterward. What, then, shall we say of sanitary analysis as an index of pollution at Burlington? Bacteriological examination revealed the abundant presence of *B. coli* in all the samples reported in Series "B," and the discharge of sewage into the lake was a matter of casual observation. Therefore no one was deceived by the nitrogen determinations. One may very properly question whether sanitary analyses may not be, under less fortunate circumstances, an actual danger to public health.

Let us now examine series "C."

SERIES "C."

PARTS PER MILLION.

No.	Nitrogen as—				Chlorine	Total Residue	Loss on Ignition
	Albuminoid Ammonia	Free Ammonia	Nitrites	Nitrates			
1	0.17	0.01	0	1.00	2.5	83	25
2	0.10	0.01	0	1.00	5.0	78	37
3	0.07	0.12	0	1.25	4.0	219	42
4	0.18	0.09	0	2.25	5.0	73	24
5	0.06	tr.	0	3.00	5.0	32	15

Records of color and odor are unfortunately absent, but from independent sources comes the assurance that none of the samples were highly colored, and the predominating odor is faintly earthy. According to the standards of interpretation, Nos. 1 and 2 were in good

condition at the time of analysis (C and D). The ammonias are low, and (A) the free-albuminoid ratio is excellent. (F) It would appear from the large amount of nitrates and the high chlorine that the water has at some time been polluted, but has become well purified. Nos. 3 and 4 are waters that have been in bad "company" (F), and the free-albuminoid ratios, especially that of No. 3, show recent pollution (A). No. 5 (see high nitrates and chlorine) appears to be an excellent example of a water purified by running in a stream-bed through a long stretch of unoccupied country below some initial seat of infection. Note the high nitrates. The truth is that all these waters were taken from unpolluted streams in the mountain districts of the Potomac drainage area in Virginia and West Virginia.

SERIES "D."

PARTS PER MILLION.

Date	Turbidity	Color	Odor	Nitrogen as—				Chlorine	Total Residue	Hardness		Free-Albuminoid Ratios	Number
				Albuminoid Ammonia	Free Ammonia	Nitrites	Nitrates			Alkalinity	Normal Hardness		
Aug. 22, 1903..	1	4	0	0.056	0.050	0.	0.025	1.73	68	46	8.3	1:1	1
Sept. 28, 1903..	40	13	0	0.341	0.055	0.	0.125	3.64	121	52	23.0	1:7	2
Oct. 24, 1903..	5	26	0	0.140	0.04	0.	0.125	5.90	130	32	33.0	1:35	3
Nov. 17, 1903..	60	51	M	0.226	0.084	0.001	0.144	5.10	144	28	33.0	1:3	4
Dec. 8, 1903..	1	9	0	0.054	0.034	0.	0.025	2.80	77	29	35.0	1:15	5

Analysis No. 1, in Series "D," indicates a practically colorless and odorless water, with nitrogen in all four forms low in amount. The chlorine, too, is low and practically, the only suspicious feature about the statement is the free-albuminoid ratio (A).

No. 2 is a turbid water of moderate color. The amount of nitrogen as albuminoid ammonia is high, but the free-albuminoid ratio ("A," last part) is 1:7. (F) Nitrogen as nitrates is high. It is not a very bad water according to the interpretation, yet there are evidences that some swamps are tributary to the point at which it was taken.

No. 3 looks suspicious because of the free-albuminoid ratio (A), the moderately high free ammonia (D) and nitrates (F), and the high chlorine.

No. 4 is a turbid, highly colored water, with a moldy odor, a bad free-albuminoid ratio (A), an appearance of nitrites, and high nitrates (F) and chlorine. It is a thoroughly "suspicious"-looking water.

No. 5 has practically the same characteristics as No. 1.

The object of introducing these tables is not so much to show the misleading character of the data as to call attention to their variations. Here are five analyses of a *normal* water, taken at the same point from a small stream draining an uninhabited wilderness. Yet only two of them possess a resemblance of uniformity, and the free-albuminoid ratios vary from those of a dilute sewage to those of a potable water. The variations in chlorine, too, are interesting, and they lead one to speculate upon the actual normal chlorine value for this region. The samples were taken from the head waters of Green River in Casey County, Kentucky.

SERIES "E."

PARTS PER MILLION.

Date	Turbidity	Color	Odor	Nitrogen as—				Chlorine	Total Residue	Hardness		Free-albuminoid Ratios	Number
				Albuminoid Ammonia	Free Ammonia	Nitrites	Nitrates			Alkalinity	Normal Hardness		
Sept. 20, 1903	20	20	0	0.272	0.130	0.009	0.225	5.6	130	65	24	1:2	1
" " "	20	17	0	0.302	0.130	0.002	0.187	5.5	128	60	14	1:2.3	2

Series "E" presents several points of interest. Both waters are of moderate color and turbidity, and have no odor. According to the standards of interpretation, No. 1 is a recently polluted water. It contains a large amount of nitrogen as albuminoid ammonia, (A) and the free albuminoid ratio is 1 to 2. Free ammonia is very high (D). Nitrites and nitrates (E and F) are both high. On the whole, the water may be said to be both recently and remotely polluted. No. 2, although somewhat similar, is superior in some respects. The free-albuminoid ratio is 1 to 2.3. Free ammonia is the same, while nitrites, nitrates, and chlorine are lower, though the last is not significantly different. The fact that in No. 2 the albuminoid ammonia is higher than No. 1 is responsible for the better ratio in the former.

One might readily infer that both samples were taken from the

same stream at the same place. "Bad" water No. 1 was taken from Kentucky River above the city of Frankfort at the water-works intake. "Bad" water No. 2 is from Kentucky River *below the Frankfort sewers.* Note that the dates of sampling are the same. The important feature of Series "E" is that here is a water, or a dilute sewage, taken below the sewers of a city of 20,000 inhabitants showing practically the same, if not a better condition, according to the interpretation standards, than another sample taken from the stream above the sewers.

Cases similar to the above are very common. Two good examples are presented in Series "F."

SERIES "F"

PARTS PER MILLION.

Date	Turbidity	Color	Odor	Nitrogen as— Albuminoid Ammonia	Free Ammonia	Nitrites	Nitrates	Chloride	Total Residue	Hardness Alkalinity	Normal Hardness	Free-albuminoid Ratios	Sampling Point
MISSISSIPPI RIVER AT BRAINERD, MINN.													
Nov. 3, 1904	14	112	2V	0.382	0.022	0	0.03	1.0	150	100	4	1:17	Above town
" " "	15	112	2V	0.382	0.040	0	0.03	1.0	142	100	4	1:9.5	Below "
Feb. 28, 1905	10	40	1V	0.256	0.051	0.002	0.04	1.4	185	161	..	1:5	Above "
" " "	10	40	1V+1M	0.300	0.040	tr.	0.04	1.8	188	162	..	1:7.5	Below "
ST. LOUIS RIVER AT CLOQUET, MINN.													
Oct. 31, 1904	17	292	2V	0.502	0.036	0	0.04	1.6	121	34	1	1:14	Above town
" " "	19	112	2V	0.302	0.032	tr.	0.08	1.8	156	98	..	1:9.4	Below "
Feb. 25, 1905	−7	112	2V	0.322	0.152	tr.	0.08	1.2	151	104	4	1:2	Above "
" " "	−7	112	2V	0.302	0.032	tr.	0.08	1.8	156	98	7	1:10	Below

SERIES "G."

PARTS PER MILLION.

Number	Turbidity	Color	Odor	Nitrogen as— Albuminoid Ammonia	Free Ammonia	Nitrites	Nitrates	Chlorine	Total Residue	Free-albuminoid Ratios	Date
1...	12	0	..	0.480	0.080	0	0.461	70	634	1:6	Mar. 5, 1898
2...	12	0	..	0.375	0.013	0	0.375	76	612	1:29	" 11, 1898
3...	12	0	..	0.656	0.104	0	.....	59	818	1:6	Apr. 9, 1898
4...	12	0.1	..	0.395	0.026	0	0.307	74	790	1:15	Mar. 11, 1898
5...	12	0	..	0.535	0.026	0	0.142	67	566	1:21	" 18, 1898
6...	12	0.1	..	1.200	0.033	0	0.472	57	850	1:36	" 25, 1898
7...	..	0.2	..	0.511	0.085	0	0.349	68	604	1:5	Apr. 1, 1898

The waters represented in Series "G" are typical of the western prairie states. In such waters large amounts of organic matter are always present even in the absolutely uninhabited regions. The samples above reported are taken from a stream that drains a large area underlaid with saline deposits, which account for the high chlorine content. Such waters absolutely controvert paragraph (C) of the foregoing interpretations. It will be noted that the free-albuminoid ratio specified in "A" (last part) is satisfied by all of the analyses. There are no nitrites, and only in Nos. 1, 3, and 7 does the amount of nitrogen as free ammonia exceed the standard set in "D." Nitrogen as albuminoid ammonia and nitrates are not high for prairie waters. Although some of the analyses look "good," the samples were all grossly polluted. The first three samples were taken from Kaw River 1.5 miles above Lawrence, Kans., and contain the residue of pollution from the sewers of Topeka. The last four samples ware taken from the same stream, but the point was 300 feet below the outlet sewer of Lawrence. It will be seen that the samples from below town present a better analytical appearance than those from above.

We will now consider ground waters. In the address above quoted there are the following statements:

> (A) . . . . It may be said that the best ground waters should certainly contain not over 0.01 milligram of nitrogen as free ammonia, or (B) over 0.02 milligram of nitrogen as albuminoid ammonia, (C) no nitrogen as nitrites, (D) not over 0.01 milligram of nitrogen as nitrates in a liter of water, and (F) chlorine not above the normal of the region. When a water contains (F) more than 0.05 milligram of nitrogen as free ammonia, and (G) 0.08 milligram of nitrogen as albuminoid ammonia, or 0.12 milligram of nitrogen as albuminoid ammonia, even if the free ammonia occurs in very small amounts, it is a sign of imperfect filtration or of subsequent pollution, and consequently such water should not be used for household purposes.

It is more difficult to determine the presence of pollution in a ground water by inspection than in a surface water, and in discussing ground-water analyses one is sometimes unable to make a definite statement concerning direct pollution. We can, for example, in the case of a surface water state that if the water is from an uninhabited region it must be unpolluted with animal waste. On the other hand, there is but one certain method of determining the healthfulness of a ground water. This method has been accepted

by chemists and sanitarians the world over as being the best test of the wholesomeness of all waters, whether from the surface or from the ground—namely, the incidence of typhoid fever and other water-borne diseases among the habitual users of such water as a beverage. Although there are on record many hundred analyses of ground waters, which would, by the interpretation standards above set forth, be classified as polluted, but which, judging from the location and all the surroundings, might be regarded as wholesome, nevertheless the basis of the statements made in the following paragraphs will rest solely upon the typhoid rate prevailing among the users of the various waters. The first series of ground-water analyses to be discussed are grouped in the following table:

SERIES "H."

PARTS PER MILLION.

Date	Nitrogen as—				Chlorine	Total Residue
	Albuminoid Ammonia	Free Ammonia	Nitrites	Nitrates		
Aug. 30, 1905....	0.044	0.136	0.008	0.152	1.80	341.2
Oct. 10, 1900.....	0.082	0.054	0.006	9.394	6.40	324.8
May 18, 1905.....	0.048	0.032	0.009	9.000	12.00	427.2
" 18, "	0.216	0.032	0.009	4.200	63.25	1042.4
May 11, 1898.....	0.058	0.062	0.007	0.600	10.00	432.8
Dec. 8, 1904.....	0.192	0.032	0	0.080	7.50	353.0

It will be seen from the above that all the waters analyzed contained more nitrogen in all the specified forms than would be allowable under the standards of interpretation above quoted. The analyses presented represent either the city supply of Rockford, Ill., or that from private wells which are largely used in that place. They are in all cases ground waters, and are similar in character to waters from various wells in that region. The writer has before him 135 analyses of well waters from Rockford, by far the majority of which present characteristics similar to those presented in Series "H." Rockford has the lowest typhoid fever death-rate of any city in the United States having a population of 30,000 or over. It will be noted in the various published tabular statements, such as that presented by Mr. George C. Whipple in the report of the Commission on Additional Water Supply for the City of New York that Rockford almost invariably stands at the foot of the list, with a death-rate of about 6 per 100,000.

Let us consider another case:

SERIES "I."

PARTS PER MILLION.

Organic Nitrogen	Nitrogen as—				Chlorine	Total Residue	Oxygen Consumed
	Albuminoid Ammonia	Free Ammonia	Nitrites	Nitrates			
2.75	0.280	0.07	0.001	0.57	7.5	368	3.73
2.86	1.100	1.100	0.001	0.24	12.0	569	3.01
2.68	0.610	0.142	0.001	0.37	6.3	400	2.65

The analyses in the above table represent the city water of Des Moines, Iowa. The first represents the water from the large well; the second, from the small well; while the third is an average of 42 analyses of the supply, all made in the year 1897. Here again comment upon the divergence of these figures with those given in the standards of interpretation is unnecessary. Des Moines, Iowa, is one of the most fortunate cities in the country from the standpoint of typhoid rates.

Series "J" contains respectively the average of analyses made from the Oconee and the Shetucket wells of the Brooklyn water supply. Throughout the entire period between 1897 and 1902 it will be noted that in neither case does the average number of bacteria exceed 50 per c.c., and there were no positive tests for coli during the entire period of investigation. Nevertheless, the nitrogen determinations, according to the above standards of interpretation, would condemn this water.

SERIES "J."

PARTS PER MILLION.

	Turbidity	Color	Odor	Nitrogen as—				Chlorine	Total Residue	Hardness		Iron	Bacteria per c.c.	Per cent positive tests for *B. coli* in c.c
				Albuminoid Ammonia	Free Ammonia	Nitrites	Nitrates			Alkalinity	Normal Hardness			
Oconee Wells . . .	1	6	0	0.20	2.65	0.001	0.01	4.8	148.7	101.0	5.0	0.57	33	0
Shetucket Wells .	10	25	0	0.015	0.402	0.012	0.01	264.2	713.5	80.6	159.4	2.02	50	0

Another example is contained in Series "J." The samples were taken from an isolated well at St. Cloud, Minn., and it must be confessed that the analyses have an unfavorable appearance, especially

by reason of the amounts of nitrites. The water, however, is absolutely unpolluted. The first sample contained two bacteria, and the second, one bacterium per c.c. Only one species was represented, which was not *B. coli.*

SERIES "T."

PARTS PER MILLION.

Date	Turbidity	Color	Odor	Nitrogen as— Albuminoid Ammonia	Free Ammonia	Nitrites	Nitrates	Chlorine	Total Residue	Hardness Alkalinity	Normal Hardness
Sept. 4, 1904.....	0	0	0	0.042	0.014	0.015	7.00	3.0	256	156	17
" 4, "	0	0	0	0.036	0.014	0.066	7.00	3.0	276	157	16

For a final example the following analysis is submitted, the expression of results being in terms of parts per million:

Free ammonia	0.012
Albuminoid ammonia	0.012
Nitrites	0.00
Nitrates	"strong"

The above water is from a well in Rochester, Minn. It will be seen that the ammonias are extremely low in amount, and the nitrites and nitrates practically absent. It is a water which conforms in all respects to the standards above quoted, yet it contained 1,570 bacteria per c.c., and an abundance of *B. coli.*

There remain for consideration the artesian or deep-seated rock waters. Examples might be cited in the support of the general contention of this paper, but it will be distinctly preferable merely to refer to a paragraph in the address above quoted, as follows:

> Unfortunately, however, in the study of artesian water perplexing chemical and bacteriological results are often obtained. In artesian waters so situated that surface pollution seems impossible, amounts of nitrogen as free ammonia, as nitrites, and as nitrates have often been found which, if occurring in ground waters, would cause them to be considered as polluted. The nitrogen of the nitrates in these waters may be due to fossil remains, and the nitrogen as nitrites and as free ammonia to the reduction of the nitrates by chemical action, as contact with iron sulphide, and the occurrence of the nitrogen as free ammonia also sometimes to some salt of ammonia existing in the strata through which the ground water passes. On this account the determination of the nitrogen content does not give as satisfactory data from which to draw conclusions as those obtained from the analysis of ground water.

The contentions of Professor Kinnicutt, set forth in the above paragraph, are supported by abundant evidence, and it constitutes as strong a statement in support of the writer's position as he himself could ever hope to draw; therefore the paragraph is submitted without discussion or amendment.

The analyses quoted in the following paragraphs are merely the chosen representatives of a great number that give the same testimony. They all show clearly the amount and condition of the nitrogenous matter, and can be used to differentiate in some small degree between a comparatively stable and an unstable form of organic matter in water. But they show further that all those finely drawn distinctions by which we are supposed to determine whether or not such organic matter is of benign or dangerous origin are too precarious to be seriously considered. In every case it is easy to find a host of discrediting exceptions; and when we go beyond the confines of New England and the country immediately thereabout, and especially when we select our samples from the South or the Middle West or Far West, those exceptions become the rule.

That real man, the lamented friend of the most of those contributing to this volume, Dr. Thomas M. Drown, found not a few places in or near New England where his standards of interpretation were useless. For example, many of us remember hearing him say that the polluted water of the Hudson above Poughkeepsie, N. Y., does not show upon sanitary analysis any traces of sewage matter. Yet neither he nor, it is believed, the most enthusiastic supporter of nitrogen determinations would accept that raw water as a beverage. In later years, not many months before Dr. Drown's death, the writer discussed with him the advisability of making an extended series of sanitary analyses upon the waters of the Lehigh River basin. Dr. Drown approved of a sanitary survey, but failed to see any promise in the analytical work. He ended his discussion of the matter by saying: "My long experience in this line of work has impressed me with many doubts concerning its value."

The practice of making sanitary analyses and of judging the potability of a water from them has cost many lives. The cases are numerous and too well known to require discussion. In really competent hands such analyses do not usually produce serious

results, because they are relegated into their proper place; but the supposedly competent hands are frequently brought to book. Let us review an instance.

In the memorable case of the State of Missouri *vs.* the State of Illinois and the Sanitary District of Chicago there was introduced into evidence the testimony of a professor of chemistry who qualified as an expert by relating all sorts of educational experience, both foreign and domestic. Cross-examination developed the following:

Q.: Taken in the abstract, without reference to anything else than the elements of pollution stated by you, to wit, free ammonia, 0.063, nitrites 0.002, albuminoid ammonia 0.552, nitrates 0.39, are those figures sufficient to warrant you in a conclusion as to the potability of the water?

A.: I think so.

Q.: What is your conclusion?

A.: It is a potable water. . . . .

Q.: Do you consider a water having the following constituents potable, namely, free ammonia 0.217, nitrites 0.013, albuminoid ammonia 0.676, nitrates 0.6?

A.: No, sir.

Q.: On what account?

A.: Because the free ammonia has gone beyond 0.2, and the nitrites are up in the second place, whereas potable water should not have free ammonia very much above 0.1; and, in fact, if the nitrites are measurable at all, we usually condemn the water; . . . .

Here is a man who gave two positive opinions concerning potability of two waters, from the bare statement of the four nitrogen determinations. He did not think it necessary to take into account the other conventional statements. There are two lamentable features of this: first, he is teaching sanitary chemistry to students in a high-grade university; second, he is only one of a large number of persons similarly situated who are addicted to precisely the same absurdities.

It is anticipated that one of the principal objections made to the foregoing discussion will be that the examples given are exceptional cases, and that a far greater number of examples can be adduced which will support the standards of interpretation; that a few exceptions do not, in science, destroy a theory, and that a great mass of data collected during past years should be accepted as the determinative basis. The cases presented for illustration are not exceptional ones, nor, indeed, are they the best that might have been selected for the purposes of this paper. If, however, we admit, for the

purposes of argument, that they may be exceptional, the contentions of the writer are not damaged thereby. It should be remembered that in making sanitary analyses we are not developing a scientific theory that must stand or fall according to the weight of cumulative evidence for or against, but we are trying to determine whether or not the use of that water will cause sickness and death. This is a positive purpose; and if it is admitted that there can be exceptions, even though they be few, the whole scheme of analytical procedure fails of that purpose. Exceptions are not predestined, and in this case cannot be guided or defined. The chemist who calls a water "good" upon the evidence presented by his nitrogen determinations has no means of knowing whether or not this water may be one of the exceptions. Supposing it be polluted like the Lake Champlain samples in Series "B," and a family or a community accepts the favorable opinion of the chemist and is stricken with typhoid fever —think you that that chemist will be justified by appearing before those bereaved relatives and reciting the fact that the great mass of evidence sustains certain bases of interpretation, and the scattering exceptions do not, from a scientific standpoint, destroy the integrity of the theory? The most befitting remark at this juncture seems to be the old proverb: "A chain is no stronger than its weakest link."

After all, perhaps the strongest indictment of the sanitary analysis is that it is unnecessary for the purposes for which it is generally used. No one will question its value in sewage experiments, but when the purpose is to determine whether or not a water be potable, there are more satisfactory ways of solving the problem than by making the conventional grind of nitrogen determinations, even though it be admitted for the moment that those determinations fulfil all the great purposes claimed for them.

It may be accepted as axiomatic that no river, upon the drainage area of which there is any population, will furnish a water fit for domestic consumption in its raw state. That this shall hold true it is not necessary that the population shall be gathered into cities and be provided with sewerage systems. Rural population, even though widely scattered, is dangerous. Wherever people live along the banks of a stream there will always be dangerous pollution. Indeed, the

natural drainage from occupied land is not always innocuous; but when this is combined with those direct and generally surreptitious pollutions, the effect is sometimes more acute than that produced by the everyday discharges from a city sewer. It will be necessary merely to recall the history of some of our classic typhoid epidemics to demonstrate this. The Plymouth, Pa., epidemic was caused by a single focus of infection upon a sparsely settled drainage area. The New Haven epidemic arose from a similar cause, and upon a drainage area not only sparsely settled, but supposedly well protected. It is especially significant, too, that the Lowell and Lawrence epidemics did not have as their immediate cause the infected sewage from cities above on the Merrimack, but, as shown by Professor Sedgwick, from one or two incidental pollutions of Stony Brook. The same principles apply forcibly to the more recent epidemics at Butler, Pa., and Ithaca, N. Y. These remarkable instances illustrate the dangers of surface-water drainage from sparsely settled countries; it is obviously unnecessary to discuss similar dangers from water into which city sewage is poured. The question whether a river water will purify itself from such discharges in a given distance below a sewer outlet does not enter even remotely into this consideration, for, assuming that the sewage discharges would be purified, the incidental pollutions above a water intake would still constitute a grave danger. In the case of the Lowell and Lawrence epidemics, for example, perfect sewage purification at Manchester, Concord, and other cities on the Merrimack above the Lowell intake would not have prevented the scourge of typhoid. Therefore it is contended that, if we accept the principle that no surface-water draining from an inhabited area is safe in its raw state for domestic consumption, we shall err, if we err at all, upon the safe side, and there is no question that we shall save lives. What, then, is the necessity for analyzing river water for pollution, if we are all agreed that it must inevitably be polluted?

Upland conserved supplies present a different phase of the question. If a drainage area contributing to a reservoir is in primeval condition with respect to population, it is generally admitted that the water must be wholesome. Why, then, make sanitary analyses to determine the presence of sewage? If it be contended that this

area may be subject to occasional malicious pollutions by visitors, etc., then the sanitary analysis does not offer any helpful solution. A single infectious intestinal discharge deposited directly in a reservoir might readily cause a typhoid epidemic, but the organic matter would not, except under very fortunate circumstances, be detected by the nitrogen determinations; and it is well to reflect that, under the usual conditions, by the time the disease had made itself manifest and attention directed to that reservoir, the infection would have passed out of the reservoir.

If the drainage area above described *does* contain population, then the danger is always impending, and we may rest upon assumptions, nearly, if not quite, as positive as those quoted above for river waters. It is quite significant in this connection to note that the Commission on Additional Water Supply of the City of New York made provision for filtration, although the upland areas proposed as new sources are sparsely settled. More recently we have read the opinions of our foremost authorities that the present Croton supply should be filtered. It is doubtful if any of those authorities would contend that the sole object of such filtration is to remove turbidity, color, and odor. It is therefore held that the sanitary analysis of upland conserved supplies is needless, because we can determine the danger by inspection far more readily and surely.

With reference to ground waters: We have interesting accounts of cases in which it is asserted that the condition of pollution was not detected by biological examination, but was revealed by sanitary analysis. If close consideration be given to the descriptions of premises that appear in these accounts, it will be seen that in every case (so far as the writer is informed) a careful man would have been justified in condemning those waters upon superficial examination, and without regard to analysis. Take for illustration the case cited by Professor William P. Mason in a paper entitled, "Interpretation of a Water Examination," which appeared in *Science*, Vol. 21, No. 539, pp. 648–53. It appears from this that there was a certain farmhouse in England, the residents of which had suffered severely from diphtheria and typhoid fever. Examination showed that the sewage discharged from the house entered into a dry-steyned cesspool, without overflow, about four yards from the well, both sunk

in gravel. In this case the chemical examination revealed the presence of an excess of chlorides and nitrates while bacteriological investigation showed nothing which would cause suspicion. This instance is cited by Professor Mason as one in which "the danger signal was held out by the chemical side of the investigation alone." The writer is of the opinion that the "danger signal" was not the findings of the analysis, but the occurrence of disease in that residence. It should have caused an immediate examination of the premises, and such examination would have revealed the fact that the sewage was discharged into a *dry-steyned cesspool*, that the well was *only four yards away* and that both were *sunk in gravel.* In the face of all our knowledge of the transmission of water-borne diseases, and in view of our decades of experience with infected wells, from historic Broad Street down to the present, why should any competent observer, with the above related facts before him, find it necessary to fuss with an ammonia still or, for that matter, with a Petri dish? Taking a broad view of the subject of well supplies, we may safely exclude all wells in questionable places; and the careful observer can usually define such places.

All of the above discussion with reference to the needlessness of sanitary analyses, when other and more expeditious methods can be used, is based upon the temporary admission that such analyses afford data whereby dangerous animal pollution can be distinguished from harmless vegetable matter. If we return now to the original contention that standards of purity, bases of interpretation, composition ratios, or by whatever name they may be called, are met with equal faith by the normal water and by the dilute sewage, and sum up the two lines of evidence, we have what the writer feels justified in regarding as an established case against the sanitary analysis as an index of dangerous water pollution.

THE VALUE OF PURE WATER.*

GEORGE C. WHIPPLE.

PREFACE.

IN order to estimate the relative value of waters which differ materially in quality, it is necessary to have some common denominator. Nothing better for this purpose has been suggested than the dollar, which in this paper is made the basis of computation. By ascertaining what different characteristics of water cost the consumers, and by finding out how much consumers are willing to pay to avoid using waters which possess certain characteristics, an attempt has been made to secure a reasonable basis of comparison. The results of this initial study are here presented. They must not be taken too seriously at present, as some of the involved assumptions have not been established beyond doubt; and with the accumulation of certain data, necessary but not as yet obtainable, the results must be somewhat modified. Yet the general conclusions ought not to be far astray, and, from a study of the best data available, the writer believes that they err on the side of conservatism rather than on the opposite side. The suggested method of calculating the value of pure water seems to be one capable of being refined to a degree where its results will be of great practical value. The lines along which the accumulation of data is necessary in order to render the method reliable will be evident from a perusal of the text.

PURE AND WHOLESOME WATER.

To define the meaning of the expression "pure and wholesome water," which is so often found in water-supply contracts, would seem to be an easy matter, after all the study that has been given to the subject in recent years; but, although everyone knows in a general way what is implied by this expresssion, yet when it comes to framing a definition in positive scientific terms, the problem is not as easy as it seems. This is not because the chemist and the biologist do not know what pure water is, but because water has so many

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attributes which have to be taken into consideration, and because these attributes vary in importance in every instance. "Pure and wholesome water" is not a substance of absolute quality. Strictly speaking, pure water does not exist in nature; all natural waters contain substances either in solution or suspension; and in proportion as these substances are present, and in proportion as they are objectionable in character, the water is impure. Definitions of pure and wholesome water, therefore, generally state what foreign substances shall not be present, or in what amounts they are permissible, instead of defining the positive qualities which the water shall possess.

Unquestionably the term "pure and wholesome water," as ordinarily used, relates to water intended to be used for drinking. Such a water must be free from all poisonous substances, as the salts of lead; it must be free from bacteria or other organisms liable to cause disease, such as the bacilli of typhoid fever or dysentery; it must also be free from bacteria of fecal origin, such at *B. coli.* In other words, the water must be free from poisonous substances, from infection, and even from contamination.* Besides this, it must be practically clear, colorless, odorless and reasonably free from objectionable chemical salts in solution and from microscopic organisms in suspension. Moreover, it must be well aërated. Color, turbidity, odor, dissolved salts, etc., may be permissible to a small degree without throwing the water outside of the definition of pure and wholesome waters. In these minor matters local standards govern up to a certain point, and it is in regard to them that differences in the judgment and experience of analysts lead to different classifications.

When it comes to using water for other purposes than for drinking, other attributes have to be considered. Hardness makes a water troublesome to wash with and to use in boilers; iron makes trouble in the laundry; chlorine corrodes pipes and makes work for the plumbers; the presence of the carbonates and sulphates of lime and magnesia affects the paper-maker, the brewer, the tanner, the dyer, the bleacher; soda causes a locomotive boiler to foam, and affects the use of the water for irrigation. All of these constituents, and others which

*By this term is meant pollution with fecal matter. Contamination must be considered as potential infection.

are not named, have to be taken into consideration in connection with a public water supply, which may be put to any of these uses.

If it is a difficult matter to define a pure and wholesome water in strict scientific terms, it is still more difficult to compare waters which differ in purity on any reasonable basis; and yet this often has to be done. Given two water sources equally available to a city for purposes of supply, both safe to drink, but one high-colored and soft, the other colorless and hard—which is the better selection? A water-works plant is to be appraised: structurally the system is a good one but the quality of the water is unsatisfactory because of its excessive color or turbidity—how much should be deducted from the value of the works because of the bad quality of the water? The water-works owned by a private company are to be purchased by the city; the city has a high typhoid fever death-rate due unquestionably to the water supply—how much less should the city pay because of that fact? A city in the West is using turbid river water—how much can it afford to pay to filter it? A city in New England is using a water so heavily laden with Anabaena that it is nauseous to drink—how much can the city afford to pay to procure a new supply? These are all practical, everyday questions which deserve answers based on scientific data.

In valuation cases, where the quality of the water supply has been unsatisfactory, the cost of filtration, or other appropriate method of purification, has been sometimes taken as a measure of the inferior quality of the water, and this amount deducted from the value of the works. In case filtration was impractical, or more expensive than securing a supply from a new source, the additional cost of such new supply has been sometimes taken as a measure of the inferior quality of the works and the amount deducted from the value of the works. Both of these methods are similar in that they contemplate the substitution of a satisfactory water for one not satisfactory.

Another method of measuring the depreciation applicable to a water-works plant because of an inferior quality of the supply would be to ascertain what the use of the impure water has cost the consumers, compared with what a pure and satisfactory water would have cost them. This method has not been used in practice, but it seems to be a reasonable one, and one which would be of more general

application than the preceding, if the data upon which it is based could be accurately determined. Unfortunately, this is not the case in many instances, but by the use of certain generalized data and assumptions, results may be secured which are of considerable use in comparing the value of waters different in quality.

The qualities of a public water supply which most affect the ordinary consumer are:

1. Its sanitary quality; that is, its liability of infection with disease germs or substances deleterious to health.

2. Its general attractiveness, or lack of attractiveness, as a drinking-water.

3. Its hardness, so far as this relates to the use of soap in the household.

4. Its temperature, so far as this relates to drinking.

Characteristics which affect industrial uses are too much a matter of local concern to be taken into account in a general discussion, although they are by no means of small account, and in some communities their importance might control. The qualities selected are to be considered as illustrative of the method rather than as a complete exposition of it.

The problem is to express these four characteristics in terms of dollars and cents to the consumer. The financial standard is certainly not the highest one for judging the quality of a water supply when the public health is concerned; human life cannot be estimated in gold dollars, and the smell of unsavory water to a thirsty man cannot be reckoned in dimes; nevertheless, the financial basis is a convenient one, and one necessarily involved in all questions which relate to public utilities.

SANITARY QUALITIES.

If the water under consideration has been used for a considerable time, the typhoid fever death-rate of the community will fairly well represent the sanitary quality of the water supply. It will not tell the whole story, but in most cases it will not lead far astray. In order to reduce this to a financial basis, it is necessary to make several assumptions.

The financial value of a human life is generally taken as $5,000,

but according to Leighton[1] it varies at different ages from $1,000 to $7,000, as shown by Table 1. It so happens that persons are most susceptible to typhoid fever near the age when their life-value is considered greatest. By combining the life-value at different ages with the age distribution of persons dying of typhoid fever, the resulting average value of persons dying from typhoid fever is found to be $4,634, which is very close to the figure ordinarily used.

The percentage mortality of typhoid fever patients is sometimes stated as 10 per cent; that is, ten cases for every death. Figures of this character are most often based on hospital records, and mild cases do not generally reach the hospitals. Studies of recent typhoid epidemics indicate that 15 to 18 cases for each death would be nearer the truth. The expense of medical treatment, nursing, and medicine, the loss of wages for a month or more, together with other attending expenses and inconveniences, would doubtless aggregate at least $100 per case, or $1,000 for the 10 cases corresponding to one death. If the estimate of $100 is considered too large, it may be answered that the excess is more than offset by the fact that there are more often from 15 to 18 cases for each death than there are 10. It may be fairly assumed, therefore, that $6,000 is a very moderate estimate of the financial loss to the community from typhoid fever for each death from that disease.

TABLE 1.

Age	Estimated Value of Human Life	Per Cent of Deaths from Typhoid Fever	Product of Columns 2 and 3
0– 5 years	$1,500	5.0	$ 7,510
5–10 "	2,300	5.9	13,570
10–15 "	2,500	7.2	18,000
15–20 "	3,000	13.1	39,300
20–25 "	5,000	16.7	83,500
25–30 "	7,500	13.2	99,100
30–35 "	7,000	9.9	69,300
35–40 "	6,000	8.0	48,000
40–45 "	5,500	5.6	30,900
45–50 "	5,000	4.0	20,000
50–55 "	4,500	3.3	15,000
55–60 "	4,500	2.6	11,700
60–65 "	2,000	2.1	4,200
65–70 "	1,000	1.5	1,500
70– "	1,000	1.9	1,900
Total		100.00	$463,480

Average value of life of persons dying from typhoid fever, $4,634.

[1] M. O. LEIGHTON, *Popular Science Monthly*, January, 1902.

TABLE 2.

EFFECT OF FILTRATION ON DEATH-RATES AT ALBANY, N. Y., AND A COMPARISON WITH TROY, N. Y., WHERE THE WATER WAS NOT FILTERED.

	Death-Rates per 100,000			Per Cent Reduction of Death Rates
	1894–98, before Filtration at Albany	1900–1904 after Filtration at Albany	Difference	
Albany				
Typhoid fever	104	26	78	75
Diarrheal diseases	125	53	72	57
Children under 5 years	606	309	297	49
Total deaths	2,264	1,868	378	17
Troy				
Typhoid fever	57	57	0	0
Diarrheal diseases	116	102	14	12
Children under 5 years	531	435	96	18
Total deaths	2,157	2,028	129	6

Remark: Filtered water was introduced into Albany in 1899. The water supply of Troy has remained practically unchanged.

Typhoid fever is by no means the only disease transmitted by contaminated water. Dysentery and various other diarrheal diseases precede it or follow in its train, and in most instances these are probably due to the same general sources of contamination as those which caused the typhoid fever, although, of course, to different specific infections. The reduction of the typhoid fever death-rate following the substitution of a pure water for a contaminated water is often accompanied by a drop in the death-rate from other diseases. Thus, if the five years before and after filtered water was introduced into Albany, N. Y., are compared, it will be seen that the reductions in deaths from general diarrheal diseases and the deaths of children under five years of age were much greater than in the case of typhoid fever. There was also a reduction in malaria, but this probably represents faulty diagnosis of typhoid fever cases before the introduction of the filters, rather than a real reduction of malaria. That the reduction of infant mortality and deaths from diarrheal diseases was not due to other conditions seems probable from the fact that in the neighboring city of Troy, where the water supply was not changed, there was no such diminution during the same period. (See Table 2.)

Hazen, in his paper on "Purification of Water in America," read

at the International Engineering Congress at St. Louis, called attention to this same fact, that after the change from an impure to a pure supply of water the general death-rate of certain communities investigated fell by an amount considerably greater than that resulting from typhoid fever alone—indicating either that certain other infectious diseases were reduced more than typhoid fever, or that the general health tone of the community had been improved. Thus, for five cities where the water supply had been radically improved he found:

	Per 100,000
Reduction in total death-rate in five cities with the introduction of a pure water supply	440
Normal reduction due to general improved sanitary conditions, computed from average of cities similarly situated, but with no radical change in water supply	137
Difference, being decrease in death-rate attributable to change in water supply	303
Of this, the reduction in deaths from typhoid fever was	71
Leaving deaths from other causes attributable to change in water supply	232

From these facts it is evident that to place the financial loss to a community as $6,000 for each death from typhoid fever due to the public water supply is to use too low a figure. It probably ought to be several times as high; but recognizing the lower financial value placed on the lives of infants, and the less serious character of the other diseases, and wishing to be as conservative as possible, for the reason that typhoid fever is not entirely a water-borne disease, $10,000 per typhoid death has been used in the calculation which follows.

Since typhoid fever is a disease which may be transmitted in other ways than by the water (as, for instance, by milk, shell-fish, or flies), it is necessary to allow a certain death-rate for these other causes, for even in a city where the water supply is perfect there may still be some typhoid fever. To establish this "normal"* is a difficult matter, but for purposes of calculation we may assume it to be determined and represent it by the letter N.

If we assume that all typhoid fever in excess of N is due to the water supply, and if we assume that the daily consumption of water is 100 gallons per capita, then letting T equal the typhoid fever death-rate per 100,000—

$(T-N)$ 10,000 = loss to the community in dollars for $365 \times 100 \times$ 100,000 gallons of water, or $D = \frac{(T-N)1{,}000}{365} = 2.75(T-N)$,

*This term "normal" must not be assumed to mean *necessary* typhoid.

where D stands for the loss in dollars per million gallons of water used.

Suppose the average typhoid fever death-rate for a community which has a somewhat polluted water supply has averaged 43 per 100,000 for a period of five years, and suppose that for this place the value of N is estimated as 15, then—

$D = 2.75\ (43 - 15) = \$76.72$ if the per capita consumption is 100 gallons. If the consumption per capita is 115 gallons, D would be $\frac{100}{115}$ of \$76.72, or \$66.71; if it were 63 gallons per capita, then D would equal $\frac{100}{63}$ of \$76.72, or \$121.77.

The value of N must be naturally subject to local variation, and in order to obtain an idea as to its probable value, a compilation of typhoid fever death-rates has been made for cities and towns in different parts of the country which use ground waters or filtered waters—that is, waters which may be considered as free from contamination.

The following is a generalized summary of them:

TABLE 3.

TYPHOID FEVER DEATH-RATES IN CITIES AND TOWNS WHICH HAVE GROUND-WATER SUPPLIES.

State	Number of Cities and Towns Averaged	Number of Years Averaged	Average Typhoid Fever Death-Rate per 100.000
Maine	2	5	6.4
Massachusetts	23	5	15.8
Connecticut	4	5	9.5
New York	13	5	24.7
New Jersey	10	1	20.5
Pennsylvania	5	1	31.8
Ohio	22	5	32.4

There is reason to believe that the higher rates given above do not correctly represent the situation, because in some instances the ground water was supplemented by the occasional use of water which may have been polluted. Proximity to a large city where the water supply is contaminated was also responsible for some of the high figures; so also was the absence of sewerage systems. Nevertheless, there seems to be a slight tendency for the typhoid fever rates to increase in the United States from north toward the south in those places where the water supply is reasonably safe. There are some exceptions to the increase southward, however. Thus, in Camden, N. J., which is supplied with a pure ground water, the typhoid rate in 1901 was only 12, and 20 in 1902.

In Fuertes' book on *Water and the Public Health* a diagram is given showing that the typhoid fever death-rates in cities supplied with ground water vary from 5 to 32 per 100,000 in America, and from 6 to 33 per 100,000 in Europe, the average being about 18 in America and 19 in Europe. It is shown also that the death-rates from cities supplied with filtered water vary from 4 to 20 in America, and from 4 to 20 in Europe, the average being 12 in both cases. Recent American data for cities supplied with filtered water show that the rates are somewhat higher than these, the average being somewhat less than 20.

Taking into consideration the best available data, it seems reasonable to place the general value of N somewhere between 10 and 25 per 100,000, with the most probable average value as 20, which figure may be used in the equation where local sanitary conditions are unknown. The value of N, however, should be varied where there is reason for doing so. Where the sanitary conditions are good 15 may be taken as a fair value. In New England it might be placed lower than in regions south of the glacial drift; in cities near the seaboard, where there is a large consumption of oysters consumed fresh from the layings, the value of N might be higher than in inland cities, where the oyster consumption is small and where fattened oysters are not used as freely; in cities where there are cess-pools, but no sewers, the value of N would naturally be higher than in cities well provided with sewers.

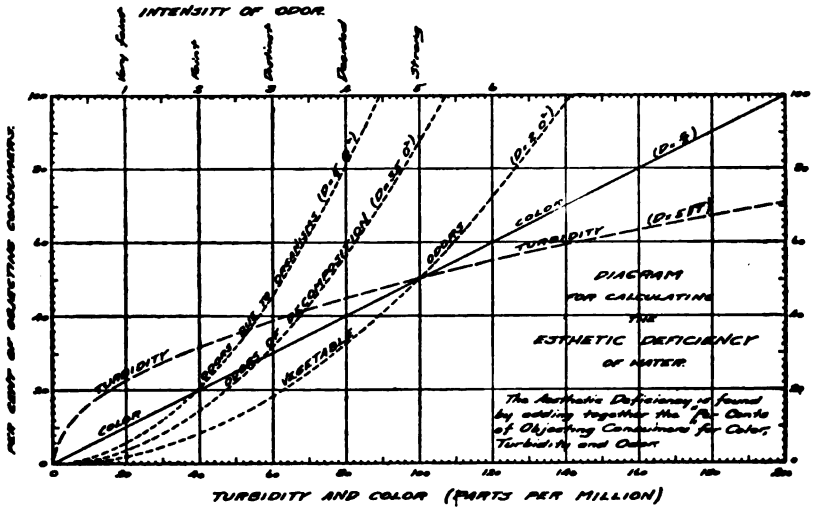
It may be reasonably expected that, as time goes on, the value of N will gradually fall, because of a general decrease of typhoid fever in the country at large, and a consequent diminution of the number of foci of infection. Statistics for twelve states, including all the New England states, New York, New Jersey, Maryland, California, Minnesota, and Michigan, show that during the last quarter of a century the general typhoid fever death-rate has fallen as follows:

TABLE 4

Year	Average Typhoid Fever Death-Rate per 100,000
1880	55
1885	46
1890	36
1895	28
1900	23
1905	21

ATTRACTIVENESS.

The analytical determinations which relate to the general attractiveness of a water are those of taste, odor, color, turbidity, and sediment. As these quantities increase in amount, the water becomes less attractive for drinking purposes, until finally a point is reached where people refuse to drink it. In order to use these results in a practical way, it is necessary to combine them so as to obtain a single value for the physical characteristics or, as they say abroad, for the "organoleptic" quality of the water. An attempt has been made by the author to obtain what may be termed an esthetic rating of the water, and the result is shown in the accompanying diagram.



This diagram, it should be said, is based almost entirely upon estimates and very little upon statistical data. It rests upon the assumption that people differ in their sensibilities, or their esthetic feelings as to the use of water. Some persons are much more fastidious than others in regard to what they drink. A water which would be shunned by one person, even though he were thirsty, might be taken by another with apparent relish. As a rule, people are more fastidious about the odor of water and the amount of coarse sediment which it contains than they are about its color and turbidity. This is perhaps natural, as a bad odor suggests decay, and decay is instinctively repugnant.

Often, however, people do not discriminate between odors which are due to decomposition and those which are not. Habit and association have much to do with a person's views as to the attractiveness of water. In New England, where the clear trout brooks run with what Thoreau called "meadow tea," few people object to a moderate amount of color, while they do object to a water which is very turbid. In the Middle West, where all the streams are muddy, it is the unknown colored waters which are disliked. People who are accustomed to well water object to both color and turbidity. With most people a fine turbidity, such as is produced by minute clay particles, is less a subject of complaint than an equal turbidity produced by comparatively coarse sediment. In the diagram an attempt has been made to reconcile these different points of view so as to put them, as well as may be, on the same footing. In this connection several series of comparisons were made.* Turbid waters were viewed through the eyes of a group of western people, who made some comparisons with color and turbid waters, while colored waters were viewed through the eyes of a group of students in New York, and *vice versa.*

The abscissæ of the diagram represent turbidity, color, and odor, as given in the ordinary water analysis.† The ordinates represent the "per cent of objecting consumers." By this is meant the proportion of the water-takers who would ordinarily choose not to drink the water because of the quality indicated by the curve, or who would buy spring water, or bottled water, rather than use the public supply, if they could afford to do so. This number would increase, of course, as the general attractiveness of the water decreased. From the curves one may calculate what may be called the *esthetic deficiency* of the water by adding together the per cents of objecting consumers for color, turbidity, and odor. If the esthetic deficiency equals 100, it indicates that the water is of such a character that everyone would object to it, and figures in excess of 100 only emphasize its objectionable character.

It will be seen from the diagram that when the color of water is less than 20, or the turbidity less than 5, only one person in ten would object to it, but when the turbidity or color is 100, one-half of the

*Acknowledgments are due to Mr. J. W. Ellms, of Cincinnati, Ohio, and Mr. Andrew Mayer, Jr., of Brooklyn, N. Y.

†See "Report of Committee on Standard Methods of Water Analysis, American Public Health Association," Supplement No. 1, *Journal of Infectious Diseases*, May, 1905.

people would object to it. It may be thought that this proportion is too low, but it must be remembered that colored waters are invariably accompanied by a vegetable odor and often by a slight turbidity, and that it is the sum of the several quantities which determines the esthetic rating.

Experience has shown that objection to color varies directly with its amount; consequently this curve has been plotted from the equation, $p_c = \frac{c}{2}$, i. e., a straight line, where p_c stands for the per cent of objecting consumers, and c for the color.

In the case of turbidity, however, small amounts count for more, relatively, than larger amounts. The equation for the turbidity curve has been taken, therefore, as $p_t = 5\sqrt{t}$, where t stands for the turbidity.

With odor, however, the opposite condition prevails; faint odors count for little, but distinct and decided odors cause much more complaint. Consequently, the per cent of objecting consumers has been made to vary as the square of the intensity of the odor expressed according to the standard numerical scale. The quality of the odor makes quite as much difference as its intensity, and for that reason three curves have been plotted, one representing vegetable or pondy odors (O_v), one representing odors due to decomposition (O_d), and one representing the aromatic grassy and fishy odors due to microscopic organisms (O_o). These curves are plotted from the following equations:

$$p_o = 5O_o^2,$$
$$p_o = 3.5O_d^2,$$
$$p_o = 2O_v^2,$$

in which O_o, O_d, and O_v stand for the intensity of the three groups of odors mentioned.

These curves represent somewhat imperfectly our present ideas as to the relative effects of color, turbidity, and odor; and on further study they are likely to be considerably modified.

It is a well-known fact that in cities which are supplied with water which is not attractive for drinking purposes, large quantities of spring water and distilled water are sold, and that consumers go to much expense in the purchase of house filters in order to improve the quality of the water furnished by the city mains. It is fair to assume

that in any community the amount of money expended for bottled water and house filters will vary in a general way according to the attractiveness of the water, although there is no doubt that the presence of typhoid fever in the community, or the fear that the water is contaminated, will greatly increase the use of auxiliary supplies for drinking. For purposes of calculation it may be assumed that the diagram just described represents this tendency to use vended waters, and that each "objecting consumer" would go to the expense of buying spring water or putting in a house filter, if he could afford it. It may be argued, also, that the poor consumer who may be unable to do this is as much entitled to satisfactory water as is the well-to-do consumer.

From a study of price-lists of spring waters sold in New York and other cities it has been found that the ordinary wholesale price of spring water is seldom more than 10 cents a gallon. In some places it is as low as 1 cent. The average is about 5 cents. To filter water through house filters costs less, but generally it is less satisfactory.

As a convenient figure for calculation, and as a most conservative one for general use, a cost of 1 cent per gallon to the ordinary consumer for an auxiliary supply of drinking-water (either spring water or well-filtered water) has been taken. In cities where the cost of procuring and distributing bottled water exceeds 1 cent per gallon, as it does in such a city as New York for example, this should be taken into account in making local use of the data. For the illustrative purposes of the present paper, and for general comparisons, the figure mentioned will serve as a satisfactory basis. The average person drinks about 1.5 quarts of water per day, and therefore one-fifth cent per capita daily may be taken as a reasonable figure for the cost of an auxiliary supply. If the entire population used such a supply, and if the daily consumption of the public water supply were 100 gallons per capita, then one-fifth cent per hundred gallons, or \$20 per million gallons, would represent the loss to the consumers due to an imperfect water supply which had an esthetic deficiency of 100. If the esthetic deficiency were less than 100, say 37, then the loss to the consumer would be $\frac{37}{100}$ of \$20, or \$7.40 per million gallons. In other words, the figure for the esthetic deficiency divided by 5 gives the financial depreciation value of the water supply in dollars per million gallons, or $D = 20\frac{p_o + p_t + p_o}{100}$.

Example: Suppose the turbidity of a water is 3, its color 65, and its odor 2f (that is, faintly fishy), because of the presence of microscopic organisms; then $D = 20\frac{12+32+20}{100} = \$12.80$; that is, the depreciation value of the water, because of its unsatisfactory physical qualities, amounts to $12.80 per million gallons.

HARDNESS.

The point at which a water becomes objectionably hard has never been exactly defined. Standards of hardness vary in different parts of the country. The ordinary person washing his hands considers the water soft if the soap will quickly produce a suds without curdling. A hardness of 10 parts per million is practically unnoticeable, and it requires a hardness of 20 or 30 parts per million to produce "curdling." Waters which have a hardness below 25 parts per million seldom cause much complaint, but when the hardness rises above 50 the water is well entitled to the appellation "hard," and above 100 it may be called very hard. In some parts of the country hardnesses of 200 or 300 are observed; these may be termed "excessive."

In 1903 a number of experiments were made by the writer to determine the effect of various degrees of hardness on the amount of soap used in washing the hands, in bathing, and in general household uses. As a result of these experiments it was found that one pound of the average soap as used in the household would soften 167 gallons of water which had a hardness of 20 parts per million. This was equivalent to about three tons of soap per million gallons, which at a cost of 5 cents per pound, would amount to $300 per million gallons. It was found also that for every increase of 1 part per million of hardness the cost of soap increased about $10 per million gallons of water softened.

All of the water used by a community is not completely softened. The number of gallons per capita per day completely softened has been estimated by different authorities all the way from 1 to 10. It will certainly be a conservative estimate to assume that one gallon per capita is thus softened. On this basis the depreciation value of water, on account of its hardness, is $D = \frac{H}{10}$, in which H equals the hardness of the water in parts per million, and D the depreciative value in dollars per million gallons.

Example: Assume the total hardness of a water to be 79 parts per million; then $D=\frac{79}{10}=\$7.90$ per million gallons.

This takes into account only the cost of soap used for domestic purposes, and does not include the incidental losses and inconveniences attendant upon the use of hard water in the household. These, if they could be expressed in terms of dollars and cents, would probably more than equal the cost of soap; therefore the above figures err on the side of conservatism.

TEMPERATURE.

Everyone knows that warm water is unpalatable. When the temperature rises above 60° F., people do not like to drink it without cooling. The relation between the temperature of the water and the per cent of objecting consumers may be represented by a curve based on the equation $p=\frac{(d-45)^2}{9}$, in which p equals the per cent of objecting consumers, and d equals the temperature of the water in Fahrenheit degrees. According to this curve, no one would object to drink a water which had a temperature of 45°, half the people would object at 66°, and all would object at 75°. If it is assumed that it takes one-half pound of ice per capita daily to cool the water used for drinking during four months in the year, and that ice costs 30 cents per 100 pounds, then the depreciation value due to temperature would be equivalent to \$5 per million gallons of public supply for 100 per cent of objecting consumers, assuming the per capita consumption to be 100 gallons daily, or $D=\frac{p}{100}\times\$5=\frac{(d-45)^2}{180}$ in dollars per million gallons, in which d = the average temperature during the four warmest months of the year. This may be considered as the depreciation value due to temperature. The temperature of ground waters seldom rises above 60° in the house taps even in summer, and in cities supplied with ground water a large proportion of the consumers do not use ice. Surface waters, on the other hand, in the latitude of New York, generally maintain a temperature of 60° or more at the house taps for at least four months of the year. The temperature factor is an important one in many cases,

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but it need not be used except when comparing surface waters with ground waters.

In a similar way it might be possible to calculate the reduced value of a water due to other objectionable characteristics, such as the presence of large amounts of iron or chlorine. Except in special cases, these would not be as important as the more obvious qualities above described, and they need not be considered in this discussion.

SUMMARY OF PRINCIPAL FORMULÆ.

Depreciation due to sanitary quality—

1. $D = 2.75\ (T - N)$.

Depreciation due to physical characteristics—

2. $D = 20\dfrac{p_c + p_t + p_o}{100}$

$$p_c = \frac{c}{2}$$
$$p_t = 5\sqrt{t}$$
$$p_o = 2\,O_v^2 + 3.5\,O_d^2 + 5\,O_o^2\,.$$

Depreciation due to hardness—

3. $D = \dfrac{H}{10}$.

Depreciation due to temperature—

4. $D = \dfrac{(d-45)^2}{180}$, in which—

D = the depreciation value in dollars per million gallons;
T = typhoid fever death-rate per 100,000;
N = typhoid fever death-rate assumed to be due to causes other than water, and which may be ordinarily taken as 20 per 100,000;
p_c = per cent of consumers who object to the color of the water;
p_t = per cent of consumers who object to the turbidity of the water;
p_o = per cent of consumers who object to the odor of the water;
c = color reading;
t = turbidity reading;

O_v = odors due to vegetable matter, expressed according to standard numerical scale;

O_d = odors due to decomposition, expressed according to standard numerical scale;

O_o = odors due to microscopic organisms, expressed according to standard numerical scale;

H = hardness of water in parts per million;

d = average temperature of water during four warmest months.

APPLICATION OF THE FORMULÆ.

It now remains to apply the principles above set forth to actual cases and see to what conclusions they lead.

EFFECT OF CONTAMINATION.

The average death-rate from typhoid fever in American cities which have more than 30,000 inhabitants is about 35 per 100,000. Applying formula (1), and assuming a value of 20 for N, then

$$D = 2.75(35 - 20) = \$41.25;$$

that is, the average depreciation value of the water supplies of our American cities, taken as a whole, is $41.25 per million gallons because of their unsanitary quality, or about $15,000 per annum for each million gallons a day of supply.

The above figure takes into account both good and bad supplies. The average typhoid fever death-rate in those cities which have reasonably good water supplies may be taken in round numbers as about 20, while in those cities which have supplies more or less contaminated it varies from this up to 40 or 60. In some of the worst cases it is more than 100 per 100,000. In Pittsburg, for example, the typhoid death-rate for several years has averaged 120. Here, according to formula (1), $D = 2.75\ (120 - 20) = \$275$ per million gallons. This is figured, however, on a per capita water consumption of 100 gallons a day. The actual consumption is about 250 gallons per capita per day; hence D should be taken as $\frac{100}{250}$ of $275, or $110 per million gallons. Each million gallons of polluted Allegheny River water pumped to Pittsburg has therefore reduced the vital assets of the community by $110. This, for a population of 350,000, amounts to $3,850,000 per year—a sum enormously greater than the cost of making the water pure.

Classifying water supplies according to their source, the following will give a general idea as to the depreciation value of various types of water from the sanitary standpoint, based on general average typhoid fever death-rates:

CHARACTER OF WATER SUPPLY.

	Depreciation Value in Dollars per Million Gallons
1. *Ground waters*, except in cases where pollution is excessive, or where wells are driven in rock or soil abounding in fissures .	$0.00
2. *Filtered waters* (assuming modern methods of construction and operation), ...	$0.00
3. *Surface waters*—	
a) Ordinary upland waters, with insignificant contamination .	$ 0.00 to $ 15.00
b) Slightly contaminated waters, with good storage in lakes or large reservoirs	10.00 to 50.00
c) River waters, slightly contaminated, little or no storage .	25.00 to 100.00
d) River waters, much contaminated, little or no storage . .	50.00 to 200.00

EFFECT OF TURBIDITY, COLOR, AND ODOR.

It has been shown that the esthetic deficiency of water depends upon three variable characteristics, which may have many different combinations; consequently, it is difficult to classify the water sup-

TABLE 5.

EXAMPLES OF WATERS WITH DIFFERENT PHYSICAL CHARACTERISTICS.

City	Source of Supply	Turbidity	Color	Odor	Per Cent of Objecting Consumers	Depreciation Value per Million Gals.
	GROUND WATERS.					
Camden, N. J....	Driven wells.....	0	1	0	0	0.00
Flatbush, L. I....	Driven wells.....	0	0	0	0	0.00
Lowell, Mass.....	Driven wells.....	0	10	0	5	1.00
	SURFACE WATERS.					
Portland, Me.....	Lake Sebago.....	1	15	2*v*	20	$ 4.00
Boston, Mass.....	Sudbury and Nashua Rivers.....	3	25	2*v*	30	6.00
Cleveland, Ohio..	Lake Erie.....	18	5	1.5*v*	30	6.00
Worcester, Mass..	Storage Reservoirs.....	2	30	3*v*	40	8.00
New York City...	Croton River	4	20	3*v*	55	11.00
Brooklyn, N. Y...	Ponds and driven wells on Long Island	3	13	1.5*g*	36	7.20
Jersey City, N. J..	Rockaway River	4	32	2*v* 1*g*	38	7.60
Watertown, N. Y.	Black River	6	70	3*v*	55	11.00
Springfield, Mass.	Ludlow Reservoir	5	27	4*g*	104	20.80
Bangor, Me.....	Penobscot River	6	65	3*v* 1*m*	59	11.80
Pittsburgh, Pa...	Allegheny River.....	64	30	3*v* 2*m*	87	17.40
Philadelphia, Pa..	Schuylkill River	150	10	3*v* 2*m*	102	20.40
St. Louis, Mo....	Mississippi River.....	200	30	3*v* 2*m*	127	25.40

Some of the above figures do not represent present conditions. For example, Watertown, N. Y., now has filtered water; St. Louis uses a chemically treated water; etc.

plies of the country on this basis. For this reason the few typical examples given in Table 5 may be more instructive than any attempt at a general classification.

It will be seen from the above figures that, while the general attractiveness of a water is of less importance than its sanitary quality, yet it is by no means insignificant. For instance, such a water as that now supplied to New York City from the Croton River has a depreciation value of $11 per million gallons, or nearly a million and a half dollars a year for a daily supply of 350 million gallons. At 4 per cent this represents the interest on about $35,000,000, a sum several times as large as the cost of filtration. An algæ-laden water like that of Ludlow Reservoir at Springfield, Mass., has a depreciation value of more than $20 per million gallons, because of its odor and turbidity. A colored water like that of the Black River at Watertown before filtration has a depreciation value of $11, while a turbid water like that of the Mississippi River at St. Louis gives $25.

In most surface waters the physical characteristics vary greatly at different times of the year. During the spring and fall, for instance, the color and turbidities may be high on account of rains, while during the summer the water may have bad odors due to microscopic organisms. The depreciation value of a certain reservoir water, calculated as above described, serves well to show this seasonal variation, as illustrated by the following figures:

TABLE 6.

SEASONAL VARIATION IN THE DEPRECIATION VALUE OF A SURFACE WATER DUE TO SEASONAL CHANGES IN TURBIDITY, COLOR, AND ODOR.

Month	Turbidity	Color	Odor	Per Cent Objecting Consumers	Depreciation of Value per Million Gals.
January	6	25	3*v*	44	$ 8.80
February	8	28	3*v*	47	9.40
March	7	27	3*v*	45	9.00
April	5	22	3*v*+	40	8.00
May	8	25	(3*v* 0.5 *Org.* 0.3*m*)	49	9.80
June	7	30	(3*v* 0.3*m*)	48	9.60
July	4	22	(3*v* 0.5 *Org.* 0.5*m*)	43	8.60
August	4	25	(3*v* 2. *Org.* 0.5*m*)	62	12.40
September	3	30	(3*v* 1. *Org.* 0.5*m*)	63	12.60
October	4	28	(3*v* 1. *Org.* 0.5*m*)	49	9.80
November	3	26	(3*v* 0.3*m*)	40	8.00
December	4	25	(3*v* 0.3*m*)	42	8.40
Average	..	..	.....	..	$ 9.53

EFFECT OF HARDNESS.

The waters of New England are comparatively soft, although in some instances the ground waters are hard. In the Middle West, on the contrary, most of the surface waters are quite hard, and in some cases the hardness is excessive. The following figures serve to give an idea of the range in the depreciation value of waters due to hardness.

TABLE 7.

State	City or Town	Source of Supply	Total Hardness (Parts per Mill.)	Depreciation Value per Million Gals.
Maine	Augusta	Kennebec River	20	$ 2.00
"	Waterville	Messalonskee River	15	1.50
Massachusetts	Boston	Sudbury and Nashua Rivers	12	1.20
"	Cambridge	Storage Reservoir	33	3.30
"	Pittsfield	Storage Reservoir	50	5.00
New York	New York	Croton River	40	4.00
"	Albany	Hudson River	64	6.40
"	Oswego	Oswego River	191	19.10
Pennsylvania	Philadelphia	Schuylkill River	179	17.90
Ohio	Toledo	Maumee River	200	20.00
"	Columbus	Scioto River	335	33.50
"	Warren	Mahoning River	578	33.50
England	London	Chelsea Company	215	21.52
"	London	East London Company	243	24.30

EFFECT OF FILTRATION.

Sanitary quality.—The following figures show to what extent the sanitary value of a polluted public water supply is increased by an efficient system of filtration:

Lawrence, Mass.—

Water supply, Merrimack River, filtered by a slow sand filter.

Population 70,000.

Water consumption, 40 gallons per capita daily.

Before filtration the typhoid fever death-rate was 121 per 100,000; since then it has been 26.

Before filtration $D = 2.75\ (121-20) \times \frac{100}{40} = \$693$.

After filtration $D = 2.75\ (26-20) \times \frac{100}{40} = \$41$.

Increase in sanitary value $= \$693 - \$41 = \$652$ per million gallons, or $665,000 per year, or $9.50 per year per capita.

Albany, N. Y.—

Water supply, Hudson River, filtered by sand filter.

Population, 95,000.

Water consumption, 165 gallons per capita daily.

Before filtration the typhoid fever death-rate was 104 per 100,000; since then it has been 26.

Before filtration $D = 2.75(104-20) \times \frac{100}{165} = \$140$.

After filtration $D = 2.75\ (26-20) \times \frac{100}{165} = \$10$.

Increase in sanitary value = \$140 − \$10 = \$130 per million gallons, or \$450,000 per year, or \$4.75 per capita per year.

Binghamton, N. Y.—

Water supply, Susquehanna River, filtered by a mechanical filter.

Population, 42,000 (approximately).

Water consumption, 160 gallons per capita daily.

Typhoid fever death-rate before filtration, 49; after filtration, 11 per 100,000.

Before filtration $D = 2.75\ (49 - 11) \times \frac{160}{100} = \$65$.

After filtration $D = 2.75(11 - 11) \times \frac{160}{100} = 0$.

Increase in sanitary value = \$65.00 per million gallons, or \$160,000 per year, or \$3.80 per capita per year.

Watertown, N. Y.—

Water supply, Black River filtered by mechanical filter.

Population, 25,500 (approximately).

Water consumption, 160 gallons per capita daily.

Typhoid fever death-rate before filtration, 68 per 100,000; after filtration, 19.5.

Before filtration $D = 2.75\ (68 - 20) \times \frac{160}{100} = \$82.50$.

After filtration $D = 2.75\ (20 - 20) \times \frac{160}{100} = 0$.

Increase in sanitary value = \$82.50 per million gallons, or \$120,000 per year, or \$4.75 per capita per year.

Illustrations like the above might be multiplied, but the four cases selected are sufficient to illustrate the general fact. It is easily seen from them that the filtration of a polluted public water supply increases to a very great extent the vital assets of a community, and the increase in most cases is many times greater than the cost of constructing and operating the works. Money paid to the doctor, the apothecary, and the undertaker is not, in one sense, a loss to a community, as it is merely a transference of money from one man's pocket to another's, but in the broader sense any loss of productive capacity or any unnecessary expenditure is a loss. Deaths from typhoid fever and from other diseases, however, represent a very material loss of the productive capacity of a community, and consequently a decrease in what may be termed the "vital assets." In the case of the city of Albany, for instance, the increased worth of the water to the city, because of its efficient filtration, amounts to \$475,000 per year, of which at least \$350,000 may be considered as a real increase in the vital assets of the city.

If in the formula $D = \$2.75\ (T - N)$ we let $T - N = 1$, then $D = \$2.75$; that is, a decrease in the typhoid fever death-rate of 1 per 100,000 causes an increase in the vital assets of the city of \$2.75 for each million gallons of the public water supply (assuming this to be

100 gallons per capita), or $0.10 per capita per year for each unit reduction of the typhoid fever death-rate per 100,000. In other words the decrease in the typhoid death-rate per 100,000 divided by 10 gives the increased vital assets of the community in dollars per capita per year. Thus in the case of Albany, above given, the reduction in the typhoid fever death-rate was 78 per 100,000. On the basis of 10 cents per capita per unit decrease, this would amount to $0.10×78×95,000 =$741,000 per year, assuming a per capita consumption of 100 gallons daily, or $450,000 for a per capita consumption of 165 gallons daily, which is the figure stated above.

Looking at the matter in another way, it may be said that the purification of a polluted water is a sort of life-insurance for the people, the value of which is equal to 10 cents per capita for each unit decrease in the typhoid fever death-rate per 100,000 which it brings about. Such a sum capitalized represents a large amount of money. In Albany, for example, where the typhoid fever death-rate has been reduced 78 per 100,000, the annual saving of life-value would be $7.80 per capita. Capitalized on the basis of an annual life-insurance premium of $17 per thousand, this would represent an insurance policy of about $460 per year for each inhabitant, or $2,300 for each head of a family.

Physical quality.—The figures of Table 8 show the effect of filtra-

TABLE 8.

City	Source of Supply	Type of Filter	Sample	Turbidity	Color	Odor	Per Cent of Objecting Consumers	Depreciation in Value	Increased Value of Water
								Per Mill. Gals.	
Lawrence, Mass.	Merrimack River	Slow sand	Raw	10	40	3t 1m	58	$11.60	
			Filtered	0	40	2v	28	5.60	$ 6.00
Albany, N. Y.	Hudson River	Slow sand	Raw	40	32	3v 1m	69	13.80	
			Filtered	2	24	2v	27	5.40	8.40
Yonkers, N. Y.	Sawmill Creek	Slow sand	Raw	6	30	1v 1m	49	9.80	
			Filtered	0	3	1v	4	0.80	9.00
Poughkeepsie, N. Y.	Hudson River	Slow sand	Raw	30	55	3v 1m	78	17.60	
			Filtered	0	30	1v	17	3.40	14.20
Binghamton, N. Y.	Susquehanna River	Mechanical filter	Raw	30	20	3v	57	11.40	
			Filtered	0	5	1v	5	1.00	10.40
Watertown, N. Y.	Black River	Mechanical filter	Raw	6	70	3v	72	14.40	
			Filtered	0	8	1v	10	2.00	12.40
Little Falls, N. Y.	Passaic River	Mechanical filter	Raw	20	35	3v	56	11.20	
			Filtered	0	8	1v	10	2.00	9.20
Brooklyn, N. Y.	Baisley's Pond	Mechanical filter	Raw	15	31	2v	52	10.40	
			Filtered	2	3	0	7	1.40	9.00

tion on the attractiveness of waters—that is, upon the aggregate effect of their physical characteristics:

The above figures do not pretend adequately to represent the conditions in any of the cities included in the list, as the analysis in each case represents only one date. They are, however, typical of what the filters in the various places are doing, and they indicate that the increased value of the water, because of its filtration, is as great as the cost of the works—in some cases it is even greater. Thus if the effect of filtration on the sanitary qualities of these waters is entirely ignored and only its effect on their physical qualities considered, the filtration of these supplies would still be a profitable undertaking from a financial standpoint. If the sanitary qualities were also considered, the advantages of filtration would be found to be many times greater. This phase of the subject has not received the consideration it deserves, and it is this topic which the writer desires especially to emphasize in the present paper.

Water-softening.—The following figures will illustrate the financial value of water-softening plants:

Winnipeg, Manitoba—	
Hardness of water before treatment	580
Hardness of water after chemical treatment and filtration	193
Reduction in hardness	387
Increased value of water due to water-softening process, per million gallons	$38.70
Oberlin, Ohio—	
Hardness of raw water	170
Hardness of raw water after chemical treatment and filtration	48
Reduction in hardness	122
Increased value of water due to water-softening per million gallons	$12.20

These figures refer only to water used for domestic purposes. If industrial uses also were considered the advantages of water softening would be still more evident.

At the present time there are not many water-softening plants in existence in connection with municipal supplies, but the advantages to be gained are very great, and are becoming appreciated by the managers of railroads and industrial establishments. With a better understanding of the practical benefits to be derived from the use of soft water, it may be confidently expected that during the next 10 years the number of municipal water-softening plants will very greatly increase.

SUMMARY.

In the foregoing paper attention has been called to the following propositions:

1. Pure water as compared with impure water has a real financial value to a community.

2. This value may be measured by determining what impure water costs the community.

3. There are three principal characteristics which affect the value of water to the general consumer—its sanitary quality, its general attractiveness, and its hardness.

4. A formula is suggested for computing the effect of the sanitary quality of water on its financial value to a community. It is based on the typhoid fever death-rate.

5. A formula is suggested for computing the effect of the general attractiveness of water on its value to consumers. It is based on the physical characteristics of turbidity, color, and odor.

6. A formula is suggested for computing the effect of the hardness of water on its value to the consumers. It is based on the use of soap in the household.

7. Considered from the financial aspect alone, and disregarding all humanitarian considerations, the filtration of a polluted water supply adds very greatly to the vital assets of a community; hence, as a mere business proposition, no city can afford to allow an impure water supply to be publicly distributed.

8. The advantages to a community of having a water supply, not only safe, but also attractive in appearance, taste, and odor, are material from a financial aspect. The increased value of many waters because of the improvement in their esthetic qualities alone justifies the cost of filtration.

9. Water-softening at present does not receive the attention it deserves at the hands of municipal authorities. The economic advantages to be gained by removing the hardness of water are so great that, in many cases, the saving to the ordinary water-consumers justifies the cost of softening water.

10. The formulæ here suggested and the detailed results derived from their use are not to be considered as of great accuracy, as the assumed data are not fully adequate. They are given merely to

show the possibility of computing the value of pure water in terms of dollars and cents, and to illustrate the financial value of filtration and justify its cost.

TABLE 9

DEPRECIATION DUE TO SANITARY QUALITY.

Values of D for Different Values of T-N in the formula $D=2.75\ (T\text{-}N)$.
Values of D in Dollars for Million Gallons.

T-N	0	1	2	3	4	5	6	7	8	9
0..	0.00	2.75	5.50	8.25	11.00	13.75	16.50	19.25	22.00	24.75
10..	27.50	30.25	33.00	35.75	38.50	41.25	44.00	46.75	49.50	52.25
20..	55.00	57.75	60.50	63.25	66.00	68.75	71.50	74.25	77.00	79.75
30..	82.50	85.25	88.00	90.75	93.50	96.25	99.00	101.75	104.50	107.25
40..	110.00	112.75	115.50	118.25	121.00	123.75	126.50	129.25	132.00	134.75
50..	137.50	140.25	143.00	145.75	148.50	151.25	154.00	156.75	159.50	162.25
60..	165.00	167.55	170.50	173.25	176.00	178.75	181.50	184.25	187.00	189.75
70..	192.50	195.25	198.00	200.75	203.50	216.25	209.00	211.75	214.50	217.25
80..	220.00	222.75	225.50	228.25	231.00	233.75	236.50	239.25	242.00	244.75
90..	247.50	250.25	253.00	255.75	258.50	261.25	264.00	266.75	269.50	272.25
100..	275.00	277.75	280.50	283.25	286.00	288.75	291.50	294.25	297.00	299.75
110..	302.50	305.25	308.00	310.75	313.50	316.25	319.00	321.75	324.50	327.25
120..	330.00	332.75	335.50	338.25	341.00	343.75	346.50	349.25	352.00	354.75
130..	357.50	360.25	363.00	365.75	368.50	371.25	374.00	376.75	379.50	382.25
140..	385.00	387.75	390.50	393.25	396.00	398.75	401.50	404.25	407.00	409.75
150..	412.50	415.25	418.00	420.75	423.50	426.25	429.00	431.75	434.50	437.25

TABLE 10.

ESTHETIC DEFICIENCY DUE TO TURBIDITY.

Values of p_t for Different Values of t in the Formula $p_t=5\sqrt{t}$.
Per Cent of Objecting Consumers.

Turbidity	0	1	2	3	4	5	6	7	8	9
0	....	5.00	7.05	8.66	10.00	11.15	12.20	13.20	14.10	15.00
10	15.80	16.55	17.30	18.00	18.70	19.35	20.00	20.60	21.20	21.75
20	22.35	22.91	23.45	23.95	24.45	25.00	25.45	25.95	26.45	26.90
30	27.35	27.80	28.25	28.70	29.15	29.55	30.00	30.40	30.80	30.90
40	31.60	32.00	32.40	32.75	33.15	33.50	33.90	34.25	34.60	35.00
50	35.35	35.70	36.05	36.40	36.70	37.05	37.40	37.70	38.05	38.40
60	38.70	39.05	39.35	39.65	40.00	40.30	40.60	40.90	41.20	41.50
70	41.83	42.13	42.42	42.72	43.00	43.30	43.58	43.87	44.15	44.44
80	44.72	45.00	45.27	45.55	45.82	46.09	46.35	46.73	46.80	47.16
90	47.43	47.69	47.95	48.21	48.47	48.73	48.98	49.24	49.49	49.74
100 ...	50.00									

Turbidity	0	10	20	30	40	50	60	70	80	90
100 ...	50.00	52.44	54.77	57.00	59.16	61.23	63.24	65.19	67.08	68.92
200 ...	70.71	72.45	74.16	75.82	77.45	79.05	80.62	82.15	83.66	85.16
300 ...	86.60	88.00	89.44	90.83	92.19	93.54	94.86	96.17	97.46	98.74
400 ...	100.00	101.22	102.46	103.68	104.88	106.16	107.23	108.39	109.54	110.67
500 ...	111.80	112.91	114.01	115.10	116.18	117.26	118.32	119.37	120.40	121.44
600 ...	122.47	123.49	124.49	125.49	126.49	127.47	128.45	129.42	130.38	131.33
700 ...	132.27	133.22	134.16	135.09	136.01	136.93	137.84	138.74	139.64	140.53
800 ...	141.42	142.21	143.17	144.04	144.91	145.77	146.62	147.47	148.12	149.16
900 ...	150.00	150.83	151.65	152.47	153.49	154.11	154.91	155.72	156.52	157.32
1,000..	158.11									

TABLE 11.

ESTHETIC DEFICIENCY DUE TO COLOR.

Values of p_c for Different Values of c in the Formula $p_c = \frac{c}{2}$.

Per Cent of Objecting Consumers.

Color	0	1	2	3	4	5	6	7	8	9
0	....	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
10	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5
20	10.0	10.5	11.0	11.5	12.0	12.5	13.0	13.5	14.0	14.5
30	15.0	15.5	16.0	16.5	17.0	17.5	18.0	18.5	19.0	19.5
40	20.0	20.5	21.0	21.5	22.0	22.5	23.0	23.5	24.0	24.5
50	25.0	25.5	26.0	26.5	27.0	27.5	28.0	28.5	29.0	29.5
60	30.0	30.5	31.0	31.5	32.0	32.5	33.0	33.5	34.0	34.5
70	35.0	35.5	36.0	36.5	37.0	37.5	38.0	38.5	39.0	39.5
80	40.0	40.5	41.0	41.5	42.0	42.5	43.0	43.5	44.0	44.5
90	45.0	45.5	46.0	46.5	47.0	47.5	48.0	48.5	49.0	49.5
100 ...	50.0	50.5	51.0	51.5	52.0	52.5	53.0	53.5	54.0	54.5
110 ...	55.0	55.5	56.0	56.5	57.0	57.5	58.0	58.5	59.0	59.5
120 ...	60.0	60.5	61.0	61.5	62.0	62.5	63.0	63.5	64.0	64.5
130 ...	65.0	65.5	66.0	66.5	67.0	67.5	68.0	68.5	69.0	69.5
140 ...	70.0	70.5	71.0	71.5	72.0	72.5	73.0	73.5	74.0	74.5
150 ...	75.0	75.5	76.0	76.5	77.0	77.5	78.0	78.5	79.0	79.5
160 ...	80.0	80.5	81.0	81.5	82.0	82.5	83.0	83.5	84.0	84.5
170 ...	85.0	85.5	86.0	86.5	87.0	87.5	88.0	88.5	89.0	89.5
180 ...	90.0	90.5	91.0	91.5	92.0	92.5	93.0	93.5	94.0	94.5
190 ...	95.0	95.5	96.0	96.5	97.0	97.5	98.0	98.5	99.0	99.5
200 ...	100.0									

TABLE 12.

ESTHETIC DEFICIENCY DUE TO ODOR.

Values of p_o for Different Values of O^s_v, O^s_d, and O^s_o in the Formula $p_o = 2O^s_v + 3.5O^s_d + 5O^s_o$.

Per Cent of Objecting Consumers.

	Odor	Vegetable Odor (O)	Odor of Decomposition (O_d)	Odor due to Organisms (O_o)
0.....	None	0.0	0.0	0.0
1.....	Very faint	2.0	3.5	5.0
2.....	Faint	8.0	14.0	20.0
3.....	Distinct	18.0	31.5	45.0
4.....	Decided	32.0	56.0	80.0
5.....	Strong	50.0	87.5	125.0

TABLE 13.

DEPRECIATION DUE TO HARDNESS.

Values of D for Different Values of H in the Formula $D = \frac{H}{10}$.

Depreciation in Dollars per Million Gallons.

Hardness	0	1	2	3	4	5	6	7	8	9
0.....	....	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80	0.90
10.....	1.00	1.10	1.20	1.30	1.40	1.50	1.60	1.70	1.80	1.90
20.....	2.00	2.10	2.20	2.30	2.40	2.50	2.60	2.70	2.80	2.90
30.....	3.00	3.10	3.20	3.30	3.40	3.50	3.60	3.70	3.80	3.90
40.....	4.00	4.10	4.20	4.30	4.40	4.50	4.60	4.70	4.80	4.90
50.....	5.00	5.10	5.20	5.30	5.40	5.50	5.60	5.70	5.80	5.90
60.....	6.00	6.10	6.20	6.30	6.40	6.50	6.60	6.70	6.80	6.90
70.....	7.00	7.10	7.20	7.30	7.40	7.50	7.60	7.70	7.80	7.90
80.....	8.00	8.10	8.20	8.30	8.40	8.50	8.60	8.70	8.80	8.90
90.....	9.00	9.10	9.20	9.30	9.40	9.50	9.60	9.70	9.80	9.90

TABLE 13—*Continued.*

Hardness	0	10	20	30	40	50	60	70	80	90
100....	10.00	11.00	12.00	13.00	14.00	15.00	16.00	17.00	18.00	19.00
200....	20.00	21.00	22.00	23.09	24.00	25.00	26.00	27.00	28.00	29.00
300....	30.00	31.00	32.00	33.00	34.00	35.00	36.00	37.00	38.00	39.00
400....	40.00	41.00	42.00	43.00	44.00	45.00	46.00	47.00	48.00	49.00
500....	50.00	51.00	52.00	53.00	54.00	55.00	56.00	57.00	58.00	59.00
600....	60.00	61.00	62.00	63.00	64.00	65.00	66.00	67.00	68.00	69.00
700....	70.00	71.00	72.00	73.00	74.00	75.00	76.00	77.00	78.00	79.00
800....	80.00	81.00	82.00	83.00	84.00	85.00	86.00	87.00	88.00	89.00
900....	90 00	91.00	92.00	93.00	94.00	95.00	96.00	97.00	98.00	99.00
1,000..	100.00									

TABLE 14.

DEPRECIATION DUE TO TEMPERATURE.

Values of D for Different Values of d in the Equation $D=\frac{(d-45)^2}{180}$.

Dollars per Million Gallons.

Temperature	0	1	2	3	4	5	6	7	8	9
40	.....	.....	.....	.....	.....	.....	....	0.022	0.005	0.089
50	0.134	0.200	0.272	0.355	0.450	0.56	0.68	0.80	0.94	1.09
60	0.25	1.25	1.61	1.80	2.01	2.22	2.45	2.69	2.94	3.20
70	3.45	3.75	4.05	4.35	4.68	5.00	5.34	5.69	6.05	6.43
80	6.81	7.20	7.60	8.03	8.45	8.90	9.35	9.80	10.28	10.75

A CONTRIBUTION TO THE GENERAL PRINCIPLES OF THE PHARMACODYNAMICS OF SALTS AND DRUGS.*

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THIS paper is a continuation of those already published,[1] which have had for their object the investigation of the means by which salts and drugs influence the processes going on in living matter, and thus produce the phenomena of stimulation and depression.

PART I. PHARMACODYNAMIC ACTION DUE TO IONS.

The cause of the pharmacological action of salts upon protoplasm has been the subject of numerous investigations, but until the development of the ionic theory these investigations had led to no further result than to show that in groups of similar metals the heavier were frequently the more poisonous. The application of the ionic theory first brought some order into this part of pharmacology. The work of the American investigators Kahlenberg and True[2] and Heald, confirmed as it has been by Krönig and Paul, Höber, True, and many others, has shown in the clearest manner that there is a close parallelism between toxicity and the state of ionization of many of the metals, so that these authors conclude that the pharmacological action of any salt solution is a function, in large measure at least, of the ions into which the salt dissociates.

This general conclusion is, in my opinion, as firmly established as is the conclusion that the chemical reactions of such solutions are due to the ions they contain. Indeed, the conclusion is a necessary result of the ionic theory, since the chemical reactions in protoplasm do not differ in nature from those going on elsewhere; and if salts enter into other chemical reactions by their ions, they probably enter also in the same manner into the reactions of protoplasm.

But while this general theory is a great step forward, it stumbles against the objection that many compounds profoundly affect proto-

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[1] MATHEWS, *Amer. Jour. Physiol.*, 1904, 10, p. 291; 1904, 11, p. 455; 1905, 14, p. 204; 1905, 12, p. 421; 1904, 11, p. 238.

[2] KAHLENBERG AND TRUE, *Botanical Gazette*, 1896, 22, p. 91.

plasm, although they do not dissociate electrolytically into ions in the ordinary sense of the term. To explain the action of such compounds as ether and organic drugs, either one must fall back upon the assumption of the action of undissociated molecules, or the idea of dissociation must be extended to cover dissociation which is not accompanied by electrical conductivity. Kahlenberg and True, and indeed nearly all observers, have adopted the theory that some action must be ascribed to undissociated molecules; but it appears to me, in view of the fact that such another kind of dissociation is well known to occur—as, for example, the dissociation of NH_4OH into NH_3 and H_2O—and also that this dissociation has been shown by Nef[1] to determine the chemical reactions of such compounds, that the alternative of the action of dissociated particles is the more probable. At any rate, it would be premature to ascribe pharmacological action to undissociated molecules until the possibilities that that action is due to the dissociated particles shall have been proved to be insufficient. In the present paper I shall deal with pharmacological action due to particles dissociated as ions, and in a subsequent paper to action due to non-ionic dissociation. The general principles which I have worked out apply primarily to ionic particles, but I think it altogether probable that they will be found to apply equally well to non-ionic dissociation, since there is in all likelihood no essential difference in kind between such dissociation as that of NH_4OH into NH_3 and H_2O and ionic dissociation. The two probably differ only in that in the one case the two electrical charges are on the same particle, whereas in ionic dissociation they are on separate atoms.[2]

While, then, it cannot be denied that some action may be referable to undissociated molecules, the clear parallelism between dissociation and pharmacological action in the case of salts, and the equally clear parallelism between non-ionic dissociation and pharmacological action in organic compounds, indicates to my mind that it is to these dissociated particles as the possible cause of pharmacodynamic action that attention should first be directed.

Assuming, therefore, that the action of salts is due in chief measure to the ions of the solution, the first question to be answered is:

[1] NEF, *Liebig's Annalen*, 1904, 335, p. 192.

[2] MATHEWS, *Biological Bulletin*, 1905, 8, p. 342. See also NERNST, *Theoretische Chemie*, 4th ed., 1903, p. 378.

What enables any ion to act at all? What makes a mercury ion, for example, so enormously more toxic than a calcium or magnesium ion? The answer to this question, as I shall now proceed to show, is, that the mercury ion has an enormously greater ionic potential than the calcium ion.

IONIC POTENTIAL AND PHYSIOLOGICAL ACTION.

In an earlier paper[1] the term "ionic potential" was suggested to designate the tendency of any ion or atom to change its electrical state. Bodländer has used the term "Haftintensität" to designate the same factor, and he and Abegg have presented evidence to show that the ionic potential is one of the chief factors in determining chemical affinity.

The idea that this property of the ions of the salt might be of importance in determining their physiological action was first suggested by my colleague, Dr. J. Stieglitz, at the meeting of the American Physiological Society in Chicago in December, 1901. At that time the importance and real bearing of the suggestion were not appreciated by me, but about a year or so later I was much struck by the fact that the arrangement of the metals according to their solution tensions, as given by Nernst, was practically the same as an arrangement in the order of their toxic actions. Stieglitz's suggestion appeared to me in a new light, and I set to work to get additional evidence that it is the ionic potential which chiefly determines the physiological action of ions. In 1904 I published results showing the remarkable parallelism between toxicity and ionic potential in the action of salts on the eggs of *Fundulus heteroclitus*. In that paper I showed that valence and ionic velocity—factors to which main importance had been attached by Hardy, Loeb, Pauli, Posternak, and which have been recently emphasized by Robertson[2]—are unimportant when compared with the importance of the ionic potential as a determining factor of toxicity. I showed also that the phenomena of stimulation of the motor nerve by salts demonstrate the same relationship over again, and in the clearest and most decisive manner. Inasmuch as I had already interpreted the phenomena of chemical stimulation of motor nerves to mean that the nerve impulse was due to a progressive coagulation of the colloids of the nerve, it was a

[1] MATHEWS, *Amer. Jour. Physiol.*, 1904, 11, p. 456.
[2] ROBERTSON, *Trans. Roy. Soc. of South Australia*, 1905, 29, p. 11.

necessary inference, if this were true, that the ionic potential must be of decisive value in determining the precipitation of colloids by electrolytes. An investigation of this possibility showed that this was indeed the case. McGuigan[1] then investigated the relation between the ionic potential and the power of salts to prevent the action of the diastatic ferment upon starch, and found here also a remarkably close agreement with the theoretical anticipations.

The theory of the importance of the ionic potential has, therefore, been abundantly confirmed. It is the more surprising that it has met with little acceptance or attracted little notice, since its general bearings are exceedingly important, involving as they do the nature of chemical affinity on the one hand, and the basis of pharmacology on the other.

Owing to the importance of the subject, the slight attention it has received, and to the fact that my own ideas have become during the course of the work more clear and definite, it seemed to me desirable that the results previously presented both by myself and by others be summarized and put in a more definite, and perhaps a more comprehensible, form, together with new observations in the same direction. Since the solution tension and ionic potential are properties with which physiologists are not generally very familiar, since they lie in another field not hitherto brought into relationship with physiological processes, I have tried to get these ideas clear at the outset.

a) General physical principles involved in chemical stimulation and toxicity.—Any physiological response to an external agent, however that response is produced, implies a change in motion or in state of the atoms, molecules, and masses composing the protoplasmic system. Now such a change in state means that work has been done in producing these movements, and this work must have been done at the expense either of the internal energy of the system itself, or of the energy of the environment—in this case of the substance causing the change. There are accordingly two possible ways in which an external agent such as a salt might produce a change in the protoplasmic system. It may itself supply the energy, in whole or in part, which is necessary to bring to pass the internal movements of the system; or it may by its presence facilitate the transference of the potential

[1] McGuigan, *Amer. Jour. Physiol.*, 1904, 10, p. 444.

energy of the system itself into kinetic. The first method of action is clear, but a word may be said as regards the second. Protoplasm, both in its chemical and physical aspects, shows many of the phenomena of false equilibrium. It is as if there were considerable differences of potential in the protoplasm itself, but these differences were unable to neutralize or equalize themselves, owing to the presence of certain resistances. It is conceivable that our ions may produce results simply by acting as conductors, or in removing resistances; acting, in other words, as catalytic agents, without specifying more in detail exactly how these act. As an example of this kind of an action I may mention the generation of the nerve impulse when a motor nerve is suddenly immersed in a salt solution, or when its cut and longitudinal surfaces are connected by a wire. In this case the wire or the electrolyte serves by its presence only to equalize the difference in potential between the two surfaces, and the nerve stimulates itself by its own energy. And any electrolyte or any conductor will accomplish this result. To what extent electrolytes may thus affect protoplasmic motions cannot be foretold, but it is certainly possible, and I think on the whole probable, that some of the actions of salts will be found to be of this nature. In such cases the energy content of the salt would be of little importance. But while it cannot be denied that some of the salt action may be of this character, few specific instances are known to me.

In the second place, salts may appear to act catalytically by means of their valence by bringing about combinations between two substances, this combination resulting in one substance hastening the decomposition of the other. The ferments, for example, may in this way be mordanted, as it were, by some bivalent ions to the substances they ferment, in the manner suggested by Henri.[1] It will, however, be apparent in this case that the power of the ion to form such combinations of the right degree of looseness from which the ferment can again escape, must be dependent on the chemical affinity of the ion. Since the chemical affinity is very probably a function of the ionic potential, this case also really brings us back to the ionic potential as a highly important factor in the ion's action.

We may now turn from these hypothetical cases to the other possi-

[1] HENRI, *Revue générale des sciences*, 1905, 16th year, p. 641.

bility in which salts affect the protoplasmic movements in virtue of their own energy content.

In this case also the action of the salt may be twofold. It may either change the whole protoplasmic system by means of the energy in the salt, or it may by a transfer of a portion of its energy to one part of the protoplasm produce such a change in the latter that energy is set free by the protoplasm itself. It is clear, in other words, that the salt may destroy the protoplasm either directly, in virtue of a great interchange of energy between itself and the protoplasm, or it may destroy it indirectly, by acting on some part of the protoplasm in such a way that its own energy destroys it, or that the normal conversion of potential into kinetic energy necessary for the continuance of the vital processes is checked.

A distinction is generally made between these two forms of destruction, in that substances acting in the first manner are said to be immediately fatal; those acting in the second manner are said to exhaust the protoplasm by over-stimulation or depression. Thus mercuric chloride in large doses probably produces an immediate coagulation and destruction of the living matter. In this case an immediate and complete change in the protoplasmic system would be produced by the transfer of energy from the salt to the protoplasm as a whole. On the other hand, mercuric chloride may destroy living matter in small doses, not by this method, but by bringing about a small change in the protoplasm, by means of which internal resistance of some kind is withdrawn or increased, and the protoplasm destroys itself. In both these cases, however, the destruction of the protoplasm is a direct result of the energy content of the salt, and salts will be poisonous according as the amount of free energy in them is great or small.

For all salts and compounds producing changes in the protoplasmic system in the two last ways the chemical composition will be of little or no importance; the sole or most important factor determining action will be the potential and amount of the energy in it. The character of the carrier of the energy, in other words, is immaterial.

The foregoing considerations may be expressed in a formula:

Poisonous action of any salt = work done by it = available energy in it = amount of energy × its potential.

In the action of salts on protoplasm we have to deal, then, with a transfer of energy from the ions to the protoplasm, or vice versa. From the general principles of physics we conclude that the physiological action of any salt solution must be a function of its energy content. The question arises how this energy content is to be measured.

It has been shown that much, if not all, of the action of salt solutions is due to the ions present. We must, therefore, measure the energy content of the ions. The total energy of the ion is composed of two factors, the free or available energy and the bound energy. It is only the free energy, or that which can be transferred to or from the ion, which is of importance in this connection.

1. *The potential factor of the free energy.*—The interchange of energy between the salt solution and the protoplasm must depend on the relative potentials of the two systems, since whether any substance can transfer energy to another depends, not on the total amount of energy in the two substances or systems, but on the potential of the energy in the two cases. The action of any salt solution is then determined by its *available* energy, and by the *available* energy in any salt is meant the product of the difference of potential between the protoplasm and the salt multiplied into the amount of energy transferred from one to the other before the potential is equalized. If the protoplasm and the ion have energy at the same potential, the difference in potential will be zero, the available energy is hence zero, the work done is zero, and the ion should produce no direct effect due to its energy content on protoplasm, though it might affect it catalytically in the manner indicated.

2. *Total free energy.*—The total free or available energy of any ion is composed of two factors, the potential energy and the kinetic energy. The *kinetic energy*, or energy of motion, will be equal to $\frac{1}{2} MV^2$. As I do not know the actual velocity of ionic movement when the potential gradient is unknown, I am unable to determine the kinetic energy. It is, in any case, generally small when compared to the potential energy, although not negligible when the latter factor approaches zero. That is, if the potential of two ions and the protoplasm are about the same, these ions may have different actions owing to differences in their kinetic energy, i. e., their ionic masses and velocities. In this paper, however, I shall consider only the potential energy factor.

3. *The potential energy of ions.*—What is the measure of the potential energy of any ion? The potential energy must be the difference in the energy content of the ion or atom in different conditions. If any substance has any available potential energy, it necessarily means that it is capable of existing in two conditions which differ in their energy content, and that it gives up energy in passing from one condition to the other. That ions and atoms do exist in such different conditions is well known. Thus the chemical differences between atomic and ionic sodium, and between ferric, ferrous, and metallic iron, are due to differences in the energy content of the atoms in different conditions. The available potential energy of the sodium atom is very much greater than that of the sodium ion, as is indicated by the fact that when the atom becomes an ion, a large amount of heat is set free.

The potential energy of the ion must be sharply distinguished from the ionic potential. The potential energy is in its turn composed of a capacity and an intensity factor; the capacity factor being represented by the amount of electricity transferred; the intensity factor, by the tendency of the ion or atom to change its state; in other words, by its stability or ionic potential. The potential energy of any ion must be measured hence by the ionic potential multiplied into the capacity. The capacity factor falls out of account if equivalent solutions are compared, since in that case each equivalent has the same quantity of electricity in it, and the differences between the actions of ions are hence due to differences in ionic potential. The question now comes down to the determination of the ionic potential.

4. *The determination of the ionic potential.*—In my earlier papers it was not clear to me how this ionic potential could be determined, so I used instead, as a rough measure of it, the solution tension of the metal. I assumed that the solution tension would be the reciprocal of the ionic potential. Inasmuch as the solution tension varies with the concentration of the salt, I used arbitrarily the solution tension of the metals in normal ionic solution.

Inasmuch as the determination of the ionic potential depends on the determination of the solution tension, it is necessary to understand the latter term, and as this may not be familiar to all physiologists, the following explanation is given:

TABLE 1.

SOLUTION TENSION IN VOLTS OF ELEMENTS IN NORMAL IONIC SOLUTIONS.

Cations				Anions	
K	−2.92	Zn	−0.493	Cl	−1.694
Na	−2.54	Cd	−0.143	Br	−1.270
Ba	−2.54	Fe″	−0.063	I	−0.797
Ca	−2.28	Co	+0.045	NO_3	−2.229
	−1.88(?)	Ni	+0.049		
Sr	−2.49(?)	Pb	+0.129		
Li	−2.32	H	+0.277		
Mg	−2.26	Fe‴	+0.314(?)		
	−1.48(?)	Cu	+0.606		
Al	−0.999	Hg	+1.027		
Mn	−0.798	Ag	+1.048		

When a plate of metal is placed in water, a certain amount of the metal passes into the water in the form of positively charged particles, so that the solution becomes positively charged, the metal negatively charged. The tendency of different metals to throw off these positive particles varies, and this tendency may be measured in so many volts if the metal is placed in a solution of one of its salts of known strength. If this is done and the difference in voltage between the metal and solution is measured, one obtains a series of values for the different metals, and these values are known as the solution tension series of the metals. The measurements are generally referred to the metals when immersed in normal ionic solutions of their salts (Table 1).

By a reference to Table 1 showing this series it will be found that potassium and sodium stand at one end of the series, these metals having in normal ionic solutions a negative voltage of 2.92 and 2.54 respectively, as compared with the solution; while at the other end are the noble metals, gold, silver, platinum, and mercury, which immersed in normal ionic solutions become electropositive to the extent of more than one volt.

The solution tension measures therefore the difference in potential between the solution which contains a known quantity of the ions of the metal and the metal itself; and it expresses the difference between the tendency of the ion to deposit itself on the metal plate, and the tendency of an atom of the plate to become an ion. It will be seen that the values of the solution tension depend entirely on the concentration of the ions in the solution, and the presence of a plate or particle of the metal. For our purposes, therefore,

it is clear that the ordinary figures given for the solution tension are not strictly applicable to the physiological conditions. We introduce into the salt solution, not a plate of metal, but a particle of protoplasm, and we wish to know what is the tendency of any ion in that solution to give up its charge in whole or in part to the protoplasm. The solution tension is not therefore a proper measure of the particular property of the ion we seek.

In previous work, in order to get comparable results, I had to adopt arbitrarily the solution tension of the metals in the normal ionic solutions of their salts, although there was no reason at all why this value, rather than the value in any other concentration, should have been taken.

It is clear that what is most important in the ion in determining its physiological action, and its chemical action as well, is not the difference of voltage between a plate of metal and any solution of its salts, but rather the difference in pressure between a single ion and a single atom of the metal. That is, it is the inherent tendency of any ion in any concentration to change into an atom of its metal. This last property has been called the ionic potential. The method of computing it is as follows:

Nernst[1] has shown that the formula which expresses the amount of work necessary to compress a gas from volume 1 to volume 2 is of very general applicability, and also expresses the amount of work necessary to transform one gram atom of a metal into one gram ion at any concentration. This formula is as follows:

$$\text{Amount of work} = L = RTln\frac{v_1}{v_2} .$$

In this formula R is the gas constant; T, the absolute temperature; v_1 and v_2 the gas volumes; and the logarithm is the natural logarithm This formula may also be expressed using pressures instead of volumes, or:

$$L = RTln\frac{p_2}{p_1} .$$

In this formula p_1 is what is known as the solution pressure of the metal, and p_2 the osmotic pressure of the ions of the metal in the solution. Instead of p_1 we may write P. By taking R and T in absolute

[1] NERNST, *Theoretische Chemie*, 1903, 4te Aufl.

units, and remembering that in all cases one gram ion carries $n \times 96{,}540$ coulombs of electricity, where n is the valence, this formula may be expressed in the form of potential in volts existing between the metal and solution,[1] i. e.:

$$E = \frac{RT}{n} ln \frac{p_2}{P} = \frac{0.057}{n} \log_{10} \frac{p_2}{P} \text{ volts.}$$

If this potential is measured directly by connecting the metal immersed in a solution of its salt through a voltmeter with an electrode of known potential, E may be measured, and then P is easily calculated. When P is once known, E may be calculated when the metal is immersed in any solution of its salts of which p_2 is known.

To determine the real ionic potential from this formula, one proceeds as follows: It is obvious that the formula expresses the amount of work done in accomplishing two different things. It expresses the sum of the work necessary to transform one gram atom of metal into one gram ion *in the same space*, plus the amount of work (negative) necessary to expand the one gram ion from this space to one liter or the space it finally occupies. It is the first of these factors which we wish to determine, since this measures the ionic potential. The formula may accordingly be written as follows:

$$L = RTln \frac{p_2}{P} + RTln \frac{p_3}{p_2} .$$

In this formula p_2 is the osmotic pressure of the positive ions of the metal when at the same concentration as the atoms of the metal; and p_3 is the osmotic pressure of the ions when at the concentration of one gram ion to the liter. Accordingly, the first term of the right-hand member of the equation measures the work necessary to transform one gram atom of the metal into one gram ion occupying the same space, and the second term measures the work done (negative) in expanding from this space to one liter. Expressing this formula in volts, and putting C = concentration in place of p, and passing to common logarithms

$$E = \frac{0.057}{n} \log \frac{c_2}{C} + \frac{0.057}{n} \log \frac{c_3}{c_2} . \qquad (1)$$

In this equation E is determined by measurement, and the second term is easily calculated. The middle term, or the ionic potential, is then

[1] *Ibid.*, p. 701.

obtained by the difference between E and the second term. For example, a silver plate in contact with a normal ionic silver nitrate solution shows a difference of potential between itself and the solution of $+1.048$ volts. $\therefore E=+1.048$ volts. c_2 is the concentration of silver atoms in metallic silver. One gram atom of silver—i. e., 107.9

TABLE 2.

THE IONIC POTENTIALS OF THE IONS OF METALS IN VOLTS.

K −2.92(?)	Cd −0.089	O −0.426
Na −2.54(?)	Fe″ +0.00	Cl −1.694(?)
Li −2.32(?)	Co +0.107	Br −1.270(?)
Ba −2.54(?)	Ni +0.112	I −0.797(?)
Ca −2.26(?)	Pb +0.179	
Sr −2. (?)	H +0.107(?)	
Mg −1.160	Cu +0.668	
Mn −0.737	Hg +1.080	
Zn −0.434	Ag +1.163	

grams of silver—occupies the space at 18° of 10.1 c.c.; i. e., the atomic weight in grams divided by the specific gravity. c_2, or the number of gram atoms of silver in one liter of silver, $=\frac{1000}{10.1}$. $c_3=1$, since the concentration of silver ions is one gram ion per liter. Substituting these values in (1),

$$1.048 \text{ volts}=0.057 \log \frac{c_2}{C}+0.057 \log \frac{10.1}{1000}$$

or

$$1.163=0.057 \log \frac{c_2}{C}.$$

There is hence a difference of potential of 1.163 volts between one atom of silver and one ion, in favor of the ion. That is, when one gram ion of silver changes into one gram atom *in the same space* $96{,}540\times1.163$ Joules of energy are set free, or since each monovalent ion carries 9.65×10^{-20} coulombs of electricity, when one silver ion changes into a silver atom at any concentration, $9.65\times10^{-20}\times1.163$ Joules of energy are set free.

It will be seen, by comparing Tables 1 and 2, that the true values of the ionic potential calculated in this way do not in most instances differ greatly from the values of the solution tension in normal ionic solutions. The heavy metals have, as a rule, an ionic potential about 0.07−0.1 volts different from the solution tension in normal ionic solutions.*

* I have not calculated the ionic potentials of Cl. Br, and I, but have used instead the figures for solution tension in normal ionic solution. It is also impossible to calculate the ionic potentials of Ca, Li, Ba, Na, and K.

A word may be said about the reliability of these figures. They depend, as will be seen, upon direct measurements of the electromotive force shown between two metals when immersed in known solutions of their salts, those solutions being in contact. This assumes a knowledge of the number of ions of metal in the salt solutions in question, and this factor is not in all cases perfectly certain. A much more serious source of possible error arises from a determination of the potential of any single metal, since it is necessary to know the potential of at least one electrode before the rest can be determined. The measurement is ordinarily made by using the calomel mercury electrode, which is supposed to have a potential of +0.56 volts. This voltage was determined by measuring the potential between a dropping mercury electrode, which theoretically should have a zero potential, and the calomel mercury electrode. Recent determinations of the potential of the calomel mercury electrode by a totally different method by Billitzer[1] give a different value. It is, therefore, impossible at present to say which of these measurements or methods gives the more reliable result. Nernst has accordingly proposed that the hydrogen electrode in normal ionic solution be regarded arbitrarily as zero, until the absolute potential is determined. I have, however, used the values as given by Wilsmore, based on the calomel electrode 0.56.

The question does not influence most of the measurements which follow, since these are based on the sum of the potentials of both anion and cation, or upon the differences between two like ions. This is a constant whatever the absolute potential, since if a certain number is added to the anion, it will be subtracted from the cation. For example, suppose it be shown by a change in the point of zero potential that the solution tension of sodium is 2.34 instead of 2.54, this increases the ionic potential by 0.2 volt; but if sodium is 2.34, then chlorine is 1.894 instead of 1.694, and this reduces the ionic potential of chlorine by 0.2 volt. The sum of the potentials of sodium and chlorine remain unchanged.

b) *The relation between physiological action and ionic potential.*—To bring out the relationship between ionic potential and toxic action, I have prepared Table 3, which shows this relationship for the toxic

[1] BILLITZER, *Ztschr. für Elektrochemie*, 1902, 8, p. 638.

action of salts on fish eggs,[1] the diastatic ferment,[2] bromelin,[3] a proteolytic ferment, and growing tips of peas and beans.[4] Table 3 shows that the chlorides of the various metals arrange themselves, as regards their toxicity, with few exceptions, in the order of the potential energy of their ions. Ions of low potential energy, such as those of sodium, lithium, magnesium, and potassium, being relatively inert, when compared with the enormously toxic action of the ions of high potential energy, such as nickel, lead, hydrogen, ferric, cupric, mercury, silver, gold, and platinum.

There is, I think, no mistaking this general parallelism, which was theoretically anticipated. By no other properties known to us can the metals be arranged in an order so closely corresponding to their

TABLE 3.

MINIMUM FATAL DOSES OF SALTS FOR VARIOUS FERMENTS AND ORGANISMS.

(V = Dilution Minimum Fatal Dose (Equivalent).)

SALT	MINIMUM FATAL DOSE (V) EQUIVALENT DILUTION						
	Diastase	Eggs of Fundulus	Cilia Volvox	Bromelin	Roots of Pisum	Roots of Zea mais	Roots of Lupinus
$Ag\ NO_3$	<100,000	100,000	300,000	110,000	204,800	300,000	300,000
$Hg\ NO_4$	.....	.....	.....	75,000	.....	.....	.....
$Hg\ Cl_2$	30,000	50,000	.....	.....	204,800	25,600	.....
$Hg\ (CN)_2$	.....	.....	.....	.....	.....	.....	51,200
$Cu\ SO_4$	.....	.....	.....	30,000	51,200	102,400	12,800
$Cu\ Cl_2$	8,333	15,000	.....	24,000	51,200	102,400	12,800
$Te\ Cl_3$	333	4,000	.....	.....	.....	.....	.....
$Pb\ (NO_3)_2$	30(?)	.....	.....	20,000	.....	.....	6,700
$Pb\ Cl_2$	.....	5,000	.....	18,500	.....	.....	.....
$Pb\ (C_2H_3O_2)_2$	.....	5,000	.....	.....	.....	.....	.....
HCl	990	3,000	.....	.....	12,800	3,200	3,200
$Cd\ (NO_3)_2$	.....	.....	.....	3,000	.....	.....	51,200
$Cd\ Cl_2$	142	12,500	500	.....	.....	.....	.....
$Ni\ Cl_2$	910	500	.....	.....	.....	.....	.....
$Ni\ SO_4$	.....	.....	.....	.....	51,200	51,200	12,800
$Co\ Cl_2$	100	250	.....	.....	.....	.....	.....
$Co\ SO_4$	.....	.....	.....	2,250	.....	.....	12,800
$Co\ (NO_3)_2$	.....	.....	.....	2,400	25,600	6,400	12,800
$Fe\ Cl_2$	.....	10	.....	.....	.....	.....	.....
$Fe\ SO_4$	.....	.....	.....	.....	.....	.....	6,400
$Zn\ Cl$	69	800	.....	.....	.....	.....	.....
$Zn\ (NO_3)_2$	.....	.....	.....	14,000	.....	.....	.....
$Zn\ SO_4$	.....	.....	.....	8,350	.....	.....	.....
$Mn\ Cl_2$	6.25	4	15	.....	.....	.....	.....
$Al\ Cl_3$	3.3	3	.....	.....	.....	.....	.....
$Mg\ Cl_2$	1	2	5	.....	.....	.....	.....
$Mg\ (NO_3)_2$	.....	.....	.....	325	.....	.....	.....
$Li\ Cl$	1.4	4	.....	1,000 (Licl)	.....	.....	.....
$Ca\ Cl_2$	4	3.5	5	.....	.....	.....	.....
$Ba\ Cl_2$	1.1	2	.....	.....	.....	.....	.....
$Li\ Cl_2$	2.5	1.5	.....	400	.....	.....	.....
$Na\ Cl$	>.3	2	.....	.....	.....	.....	.....
$K\ Cl$	>.3	1.3	4(?)	100	.....	.....	.....
$Na\ NO_3$	.....	.....	.....	1,600(?)	.....	.....	.....
$K\ NO_3$	.....	.....	.....	100	.....	.....	.....

[1] Authors' results.

[2] Caldwell.

[3] McGiugan, *loc. cit.*

[4] Kahlenberg and True, *loc. cit.*; Heald, *loc. cit*

toxic action. For example, suppose it was attempted to classify them in the order of their ionic weights. It will be seen that no parallelism exists between toxic action and ionic weight, since we have nickel, atomic weight 58, lead, atomic weight 200, hydrogen, atomic weight 1, and copper atomic weight 32, following each other closely. Furthermore, attention may be called to the enormous difference in toxicity, existing between the same atom when carrying two different quantities of potential energy. Ferrous iron has as an ion very little potential energy compared with ferric iron, and it is enormously less poisonous than the latter.

It will be noticed also that in each of these cases certain metals come out of their proper order of toxicity and potential. In the case of *Fundulus* eggs, cadmium is considerably out of its proper place, whereas it follows the rule in the case of diastase; for diastase, lead is quite out of its position; for bromelin, the great exception is barium, which is very toxic. The causes of these special and sporadic exceptions will be taken up later.

TABLE 4.

MINIMUM FATAL DOSE AND IONIC POTENTIAL OF ANIONS.

(Eggs of *Fundulus heteroclitus*.)

Salt	V (Min. Fatal Dose)	Anionic Potential
$NaNo_3$	2.0	−2.229 (?)
NaCl	2.0	−1.694 "
NaBr	2.7	−1.270 "
NaI	4.0	−0.797 "
$NaBrO_3$	11.0	−0.727 "
$Na_2C_2O_4$	35.0	−0.109 "

If now we turn to the negative ions, or anions, a similar parallelism is shown to exist between toxicity and potential energy content. Thus chlorine is in all cases far less toxic than iodine, which has twice as much potential energy. The oxalates and cyanides and sulphites are the more toxic, the greater their available energy content. Unfortunately, our knowledge of the potentials of the anions is less exact than our knowledge of the potentials of the cations, so that it is impossible to follow the correspondence in detail; but sufficient is shown to prove that the same correspondence between toxicity and potential energy exists here as in the cations.

c) The quantitative relationship between ionic potential and the minimum fatal dose.—It was shown at the outset that the amount of

work any ion can do must depend upon its available potential energy; i. e., upon the product of the difference of potential between the protoplasm and the ion multiplied into the quantity of energy transferred before the potentials were equalized. Of two positive ions holding different quantities of available potential energy, that which has the more energy can do the more work. The total amount of work any number of positive ions can do will depend on the concentration of the the ions and amount of available potential energy in each ion. If we take as a standard a certain amount of work—let us

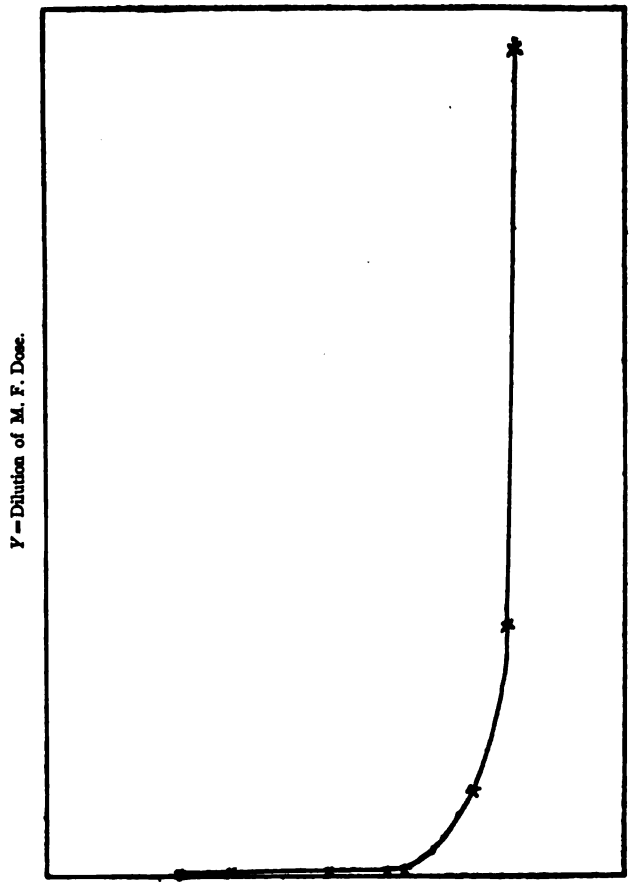

FIG. 1. Ordinates represent the dilution of the minimum fatal dose Abscissæ represent the cation potential.

say the amount of work just sufficient to kill protoplasm in unit time, say 24 hours—the concentrations of the two ions which will just accomplish this work must stand in some numerical relation to their potential energy content. What is that relation?

To bring out this relationship, I have plotted the curve in Fig. 1, which expresses the relationship between the ionic potential of the cation and the dilution (V) of the minimum fatal dose. By dilution is meant the number of liters of solution containing one gram equivalent of the salt. From an inspection of this curve it will be seen that we are dealing with a logarithmic function; that is, the dilution increases enormously in a logarithmic ratio to the ionic potential. The dilution, for example, increases 100,000 times while the ionic potential increases about five times.

By inspection of this curve one may write the general equation:

$$\log_{10} V = KE \quad . \qquad 1$$

In this formula E is the ionic potential, V the dilution, and K the constant of proportion. If, however, we place $\frac{\log V}{E} = K$, it will be seen that this formula is not in the right form, since E for some ions is zero. Nor does the formula $\log V = \frac{K}{E_a + E_c}$ give constant results. In this formula $E^c + E^a$ represents the sum of the potentials of the anion and cation. Nor could it be anticipated that such a formula would give a proper result, since what we have to express is the difference of potential between the salt and the protoplasm, and this formula expresses only the relation between minimum fatal dose and the absolute potential of the various ions. Before setting up any theoretical formula, it is necessary to get clearly in mind just in what the difference in potential between the protoplasm and the salt consists.

d) Derivation of a theoretical formula expressing the relationship between minimum fatal dose and the available potential energy of salt solutions.—We have now found out how to measure the potential of the potential energy of the salt solution; it is necessary that we discover also how the potential of the potential energy of the protoplasmic system is to be determined, since our formula involves both these factors, the available potential energy being the difference between the potentials of the salt and the protoplasm.

The protoplasmic system is made up of masses of proteid matter in equilibrium apparently with particles of the same proteid in solution. It may be regarded as a two-phase colloidal system. We may assume, in the light of the investigations of the past five years, that changes in the protoplasmic activity are due, in part at least, to changes in the state of these colloidal particles and masses, and that the salts are affecting vital processes in part by producing such changes. What we have to compare, then, in the first instance, is the potential of the energy of the protoplasmic colloids with the potential of the potential energy of the ions of the salt solution. We have, therefore, to get a clear idea of the relationship of salts to the precipitation and solution of colloids.

Since the protoplasmic colloids are for the most part composed of albumin in combination with other radicles, it is to the albuminous or proteid colloids to which attention may first be directed. The work of Kossel and Fischer has cleared up the structure of the albumin molecule, which has been shown to be a polymer of amino acids. As a result, albumin or proteid is shown to be both acid and basic; that is, it is capable of uniting with metals to form true salts, and also with acids through the amino group.

If common egg albumin is dissolved in alkaline solution, it exists as sodium albuminate; if it is dissolved in hydrochloric acid, it exists as albumin chloride. Sodium albuminate dissociates electrolytically, owing to the high dissociating power of sodium (that is, its high solution tension), and Na^{+} and $\overline{\text{albumin}}$ ions are formed. Similarly, owing to the high dissociating power of the chlorine, albumin chloride dissociates into $\overset{+}{\text{albumin}}$ and $\overline{Cl}$ ions. This dissociation results in giving the albumin—that is, the colloidal particle—a positive or negative electric charge. It is possible to change the sign of the charge on the albumin particles by making an alkaline solution sufficiently acid. This is brought about in the following way: By adding acid to the alkaline albumin the highly dissociated sodium compound is replaced by the slightly dissociated hydrogen compound. The result of this is that the charge on the colloid is neutralized, undissociated albumin is formed, and if the concentration of the albumin is sufficiently great, precipitation will occur. If one continues to add

acid, the albumin combines with the hydrochloric acid and goes over into the chloride. This at once dissociates the chlorine ion as a negative ion, and the albumin becomes the cation. It will, however, be clear that, although in this case the albumin is mainly electropositive, yet it is an acid, and the ionization of its hydrogen is not completely suppressed, although it is reduced to a very small amount. This means that here and there are albumin particles which are at one spot electronegative, since they dissociate hydrogen, and in another spot electropositive, since they dissociate chlorine. We have some amphoter or twin ion colloids, in other words. The evidence of the existence of such ions will be taken up on p. 104.

I make this explanation at such length because it does not appear to be clear to many that the albumin colloids owe their charges to processes of ionization, just as any salts owe their charges to these processes. Thus several writers have assumed that the charge was owing to the salt introduced. The ion of the salt introduced which moved fastest was supposed to bury itself in the colloid particle, and thus give its charge to it. This hypothesis is quite unfounded and undoubtedly erroneous.

The proteids, therefore, in protoplasm exist as salts, and dissociate sodium or other metallic ions, and chlorine and other anions, and the colloids thus become charged. Some proteids here and there dissociate both positive and negative ions. The colloids in protoplasm are undoubtedly in the condition of a saturated solution, and we have an equilibrium between dissociated and undissociated colloids, in solution, and undissociated and dissociated precipitated colloids.* It has been shown, furthermore, that the state of solution and the fineness of subdivision of the colloidal particles depend on the number of free electrical charges on their surfaces. The greater the number of similar charges, the greater the solubility of the colloid.

In determining the potential of the potential energy of the colloid, we have, then, just the same factors to consider as in the salts, since the colloids are salts. The potential of the energy content of the colloid solution must be determined by the ionic potentials of the ions into which it dissociates, for example, the potentials of sodium and albumin.

*As a possible example of a dissociated insoluble colloid, fibrin which has been in acid may be mentioned. This fibrin dissociates hydrogen, but the hydrogen ion is unable to move away from the fibrin. The condition is very similar to the double layer at the surface of an electrode.

In studying the action of any salt on an albumin solution, the real question to be answered is: What will be the result upon the solubility and state of the colloid of replacing the ion already in combination with the proteid by some other ion containing a different amount of potential energy? We have to know, therefore, before we can answer the question of the action of any salt on a colloid, what the ion is which is already in combination with it. This is a very important point, which is frequently overlooked in studying the action of salts.

To get a clear idea of what happens when a salt is added to an albumin solution, let us consider first, the condition of affairs in the sodium albumin solution in which the proteid exists as Na + albuminate. As the colloid stands in a saturated solution, it is in a condition of equilibrium. The sodium ion has separated a certain distance from the albumin ion. The distance it moves depends, no doubt, on several factors, but the most important will probably be its tendency to go into solution—i. e., its solution tension.* The positive ion of sodium repels a negative charge with a power equal to 2.54 volts. What the negative solution tension of the albumin ion is, unfortunately is unknown. The effect of the positive charge on the sodium will be, of course, to neutralize the negative charge on the albumin, but, owing to the fact that the sodium repels the negative charge and holds its positive charge so firmly, it is unable to neutralize it, and the colloid remains in solution. We have, in other words, an equilibrium between dissociated and undissociated sodium albuminate.

The question, then, to be solved is this: What effect will it have on the solubility of the colloid if we replace the sodium ion by another ion containing a different quantity of potential energy, i. e., having a different ionic potential? One of two results may be anticipated: either the dissociation will increase and the colloid go more completely into solution, or it will diminish and the colloid be more or less completely precipitated.

If we introduce an ion of higher potential than sodium, evidently the state of equilibrium can no longer be the same. Energy will pass from the positive ion to the albumin, and will in some degree hold or neutralize its negative charge. We may imagine that the new

*Many facts indicate that one of the most important factors in determining the ionization of salts is the ionic potential of its ions. For example, compare the ionization constants of the iodide, chloride, and bromide of mercury or silver; or compare the ionization of silver and sodium nitrates.

ion no longer can move so far from the albumin, and in consequence more nearly neutralizes its charge. The result is that the surface of the colloidal particles will be reduced, the surface tension will be increased and the colloid will be less stable. In another way of putting it, the dissociation is somewhat reduced and consequently some of the colloid tends to precipitate. If, in fact, the ion used to supplant the sodium has a sufficiently high potential, it will practically not leave the colloid at all, the dissociation will be greatly reduced, the negative charges almost neutralized, and precipitation will occur. If the ionic potential of the introduced ion is still higher, it may oxidize the colloid; i. e., an actual exchange of charges will take place between the albumin and the ion.

If, however, an ion of lower potential is introduced in place of the sodium, the reverse of these processes will take place. Ionization will be increased, the negative charge will be freer, and the solubility of the colloid will be greater. This will be the case if potassium is substituted for the sodium, provided that potassium has a lower ionic potential than sodium, as is generally assumed, and that no other factors come into play.

It is, therefore, clear that the effect of any salt upon a saturated colloidal solution of albumin in which the albumin is electronegative will depend chiefly upon what ion is in combination with the colloid when the salt is introduced.

The quantitative differences in the effects of different salts must depend upon the differences in the ionic potentials of the ion in combination with the proteid and that substituted for it.

So far, we have considered only the rôle of the positive ion. That of the negative ion is also of importance, but somewhat more difficult to picture to ourselves. We may, however, look at it in this way. The different negative ions introduced have different tendencies to deposit on the colloid, and give up their negative charges to it. This tendency is measured by the ionic potential of the ion. If the negative ion does deposit, it will tend to increase the negative charge on the colloid, and hence to dissolve it. The higher the ionic potential of the anion introduced, the greater must be its dissolving action on the colloid, since the greater will be its tendency to give its negative charge to the albumin. If the ionic potential of this ion is

lower than the albumin, it will have an opposite or precipitating action, since then the colloid will tend to give up its charge to it.

These conclusions are confirmed by my results on sodium albuminate, and those of Osborne and Harris on edestin.

From these general considerations the conclusion may be drawn that the precipitating action of the salt on the colloid will be proportional to the difference between the ionic potential of the positive ion already combined with the colloid and that substituted for it; and that the dissolving action of the anion will be proportional to the difference in potential of the anion of the colloid and that we introduce; or

$$\text{Precipitating action} = E_{c\ \text{salt}} - E_{c\ \text{colloid}}.$$
$$\text{Dissolving action} = E_{a\ \text{salt}} - E_{a\ \text{colloid}}.$$

In this formula $E_{c\ \text{salt}}$ is the ionic potential of the cation of the salt introduced, and $E_{c\ \text{colloid}}$ that of the cation of the colloid. $E_{a\ \text{salt}}$ and $E_{a\ \text{colloid}}$ are the values for the anions.

Since these two actions are mutually antagonistic, the actual action of the salt will be equal to the difference between them, or

$$\begin{aligned}\text{Actual action} &= \text{precipitating} - \text{dissolving action}\\ &= E^{\text{i}}_{c\ \text{salt}} - E^{\text{ii}}_{c\ \text{colloid}} - E^{\text{iii}}_{a\ \text{salt}} + E^{\text{iv}}_{a\ \text{colloid}}\\ &= (E^{\text{i}} - E^{\text{iii}}) - (E^{\text{ii}} - E^{\text{iv}})\ .\end{aligned}$$

If the result is positive, the salt should precipitate; if it is negative, it should dissolve the colloid. If it is zero, the salt should not affect the colloid except by mass action or by action on the water. In other words, *the actual action of any salt on a colloid in solution will be proportional to the difference between the ionic potentials of the ions of the salt, minus the difference in ionic potentials of the ions of the colloid.*

Let it be assumed that the logarithm of the dilution of the least precipitating concentration, or the logarithms of the dilution of solutions of equivalent dissolving power, are proportional to the actual action of the salt.[1] This gives the following equation:

$$\log V = K[(E^{\text{i}} - E^{\text{iii}}) - (E^{\text{ii}} - E^{\text{iv}})] + \text{const.} \qquad (2)$$

Comparing two salts with the same sign of action, i. e., dissolving or precipitating.

$$\log V_1 = K[(E^{\text{i}}_1 - E^{\text{iii}}_1) - (E^{\text{ii}} - E^{\text{iv}})] + \text{const.}$$
$$\log V_2 = K[(E^{\text{i}}_2 - E^{\text{iii}}_2) - (E^{\text{ii}} - E^{\text{iv}})] + \text{const.}$$
$$\log \frac{V_1}{V_2} = K[(E^{\text{i}}_1 - E^{\text{iii}}_1) - (E^{\text{i}}_2 - E^{\text{iii}}_2)]\ . \qquad (3)$$

[1] See Fig. I, p. 96.

That is, *the logarithm of the ratio between equivalent precipitating concentrations of two salts, divided by the difference between the differences of potentials of the ions of the two salts ought to give a constant.**

We thus have for the first time a formula for application to protoplasm which states clearly at the outset that the effect of any salt solution on the protoplasm will depend upon what ions are already in combination with the protoplasm. In other words, if we supplant most of the ions in any cell by sodium, and then apply calcium chloride, the effect will be different from that obtained if calcium chloride is applied before the sodium chloride. Furthermore, the same salt will act differently on different cells, if only those cells have different ions in them. Both of these necessary conclusions of the theory have been established by observation. To make this perfectly clear, the difference in potential between the protoplasm and the salt solution which we started to measure is the difference in potential between the systems ionized colloid—ionized salt.

However, this formula cannot be applied directly as it stands to protoplasm as a whole, because it only applies to colloidal solutions in which the colloids are all of one sign. In protoplasm, however, it is certain that we have colloids of both signs and very probably amphoter colloids; i. e., colloids which are both positive and negative at different parts of the molecule.† We probably have, in other

* This formula may, I think, be substituted with advantage for that of the tension coefficient. It is in reality the numerator of the tension coefficient.

†A number of facts speak for the presence of such twin ions in colloidal albumin solutions and in protoplasm. For example, if egg albumin is dialyzed nearly free from salts, and then coagulated so as to form a weakly alkaline colloidal solution of albumin, and if this solution is then made acid, it is well known that the albumin becomes predominantly electropositive. That is shown by the colloid migrating slowly to the cathode in an electric field, and also by the combining power of the albumin, since it now combines readily with picric and other acids to form albumin picrate, tannate, and so on. Nevertheless, if the solution is not too acid, it will combine still with the metals in some measure. This is undoubtedly due to the composition and character of the albumin. The alkali albumin first obtained by heating is a salt. When the albumin has acid added to it, there is formed, in the first instance, the free acid of the albumin, which is not much dissociated. In addition, the acid is added to the amido-group, and in an excess of acid hydrolytic decomposition being greatly reduced, the dissociation takes place so as to make the albumin predominantly electropositive. However, the ionization of the acid is not entirely prevented, although it is greatly reduced, so that in some places we must have some hydrogen ions being formed, leaving the albumin electronegative at certain places. It is probably this small percentage of hydrogen ions which can still be replaced by the metals. I found that, as a matter of fact, the heavy metals mercury and copper, although they would not in themselves cause a precipitate if the solution were sufficiently acid, yet they rendered the albumin far more easily precipitated than it was before. This is to be anticipated on our view that the colloidal particles are in some places negative and in other places positive. Not only does this appear to be the case for albumin, but in protoplasm there is also reason for believing that both ions of the salt are actually bound by the protoplasm, and especially in *Fundulus* eggs. It will be remembered that Du Bois Raymond long ago assumed that such polarized particles might exist in protoplasm.

words, ions like that in Fig. 2. As a matter of fact, if we try to apply this formula to the results on toxicity, a discrepancy between the response of protoplasm and colloidal albumin to salts is at once noticed. I have already called attention to this discrepancy. The discrepancy is this: While in the colloidal solutions the opposite action of the ions, dissolving and precipitating, is clearly apparent, and that opposite action is proportional to the ionic potentials of the anion and cation respectively, for protoplasm in general and for the ferment studied by McGuigan a different relationship is seen in that, instead of the positive ion counteracting by its energy content the negative ion, as it ought to do on the theory developed, a summation of effects is noticed. The iodides of the metals, instead of being less poisonous than the chlorides, as they should be, are more poisonous. The explanation of these facts is to be sought on the basis of the differently charged colloids present.

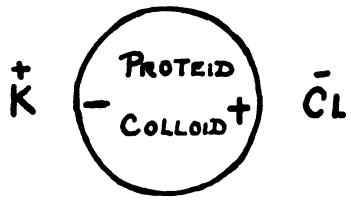

FIG. 2. Illustrating a colloidal twin ion dissociating at one place K, and at another Cl, leaving the colloid both negative and positive at different places.

If these amphoter colloidal particles exist in protoplasm, or if we are dealing in protoplasm with a mixture of both negative and positive colloids, each ion will tend to precipitate. In the twin ions, if potassium is replaced by an ion of greater potential energy, the proteid will tend to be precipitated, since the negative part will be partially neutralized; and similarly if chlorine is replaced by an anion of greater potential. Both ions, therefore, will exert an action in the same direction, and there will be a summation of action in this case instead of a difference. The formula for toxicity would become:

$$\begin{aligned}\text{Total action} &= (E_{\text{cation salt}} - E_{\text{cation colloid}}) + (E_{\text{anion salt}} - E_{\text{anion colloid}}) \\ &= (E_{\text{cation salt}} + E_{\text{anion salt}}) - (E_{\text{cation colloid}} + E_{\text{anion colloid}})\ ;\end{aligned}$$

or, writing E'_c and E'_a for the ionic potentials of the anion and cation of the salt introduced, and E_c and E_a for the ionic potentials of the ions bound to the colloid,

$$\begin{aligned}\log V^{\text{i}} &= K[(E^{\text{i}}_c + E^{\text{i}}_a) - (E_c + E_a)] + C \\ \log V^{\text{ii}} &= K[(E^{\text{ii}}_c + E^{\text{ii}}_a) - (E_c + E_a)] + C\end{aligned} \tag{4}$$

$$\log \frac{V^{i}}{V^{ii}} = K(E_c^{i} + E_a^{i} - E_c^{ii} - E_a^{ii}) \tag{5}$$

$$\frac{1}{E_c^{i} + E_a^{i} - E_c^{ii} - E_a^{ii}} \log \frac{V^{i}}{V^{ii}} = K \ . \tag{6}$$

That is, the logarithm of the ratios of the dilution of the minimum fatal doses of two salts, divided by the difference of the sums of the ionic potentials of the two salts, is a constant.

This formula is very similar to that derived by me empirically from a study of the dilutions of the minimum fatal doses of salts toward the eggs of *Fundulus heteroclitus*. The empirical formula was

$$V_a = \frac{V_o}{2^{\frac{E_a - E_o}{0.15 + 0.02\,E_a}}} .$$

In this formula E_a and E_o were the decomposition tensions of the salts.

If we take instead of 2 the base of the Naperian logarithms 2.718, and instead of $\frac{1}{0.15 + 0.02E_a}$ we write K, this goes over into the form

$$V_a = V_o 2.718^{-K(E_a - E_o)} \ .$$

Taking natural logarithms

$$\log V_a = \log V_o - K(E_a - E_o)$$

$$\log \frac{V_a}{V_o} = -K(E_a - E_o) \ .$$

This, in other words, is the same expression as that already derived, using the decomposition tension; i. e., the sum of the solution tensions of the ions, in place of the sum of the ionic potentials.

The formula may also be derived in another way. If V is the dilution of the minimum fatal dose, and if we let X represent the difference between the sum of the ionic potentials of protoplasmic ions and salt ions, obviously from the form of the curve $\frac{dv}{dx}$ varies with its position on the curve, that is with V

$$\frac{dv}{dx} = KV \ ,$$

$$\therefore \log V = KX + C = K(E_c^{i} + E_a^{i} - E_c - E_a) + C \ .$$

An application of this formula to the results of McGuigan and myself give the following values for K. In each case it is assumed

that the original ions in combination with the colloids are K and Cl.*

TABLE 5.

Salts Compared	$\frac{1}{E'_c+E'_a-E''_c-E''_a}$	$\log \frac{V_1}{V_2}$	K
$CuCl_2 - MnCl_2$	$(1.403)^{-1}$	3.1249	2.23
$HgCl_2 - MnCl_2$	$(1.816)^{-1}$	3.6812	2.03
$NiCl_2 - MnCl_2$	$(0.848)^{-1}$	2.163	2.55
$AgNO_3 - HCl$	$(0.8859)^{-1}$	2.0044	2.26
$CuCl_2 - CdCl_2$	$(0.7567)^{-1}$	1.7655	2.33
$NiCl_2 - CdCl_2$	$(0.201)^{-1}$	0.8035	3.997
$CdCl_2 - MgCl_2$	$(1.072)^{-1}$	2.1553	2.010
$HgCl_2 - MgCl_2$	$(2.241)^{-1}$	4.477	1.998
$CuCl_2 - MgCl_2$	$(1.828)^{-1}$	3.9208	2.145
$ZnCl_2 - MnCl_2$	$(0.302)^{-1}$	1.0430	3.454
$ZnCl_2 - NiCl_2$	$(0.546)^{-1}$	1.1201	2.051
$HgCl_2 - CoCl_2$	$(0.972)^{-1}$	2.4771	2.548
$HgCl_2 - CuCl_2$	$(0.4121)^{-1}$	0.5563	1.35
$CoCl_2 - MnCl_2$	$(0.843)^{-1}$	1.2041	1.428

Mean value of K 2.23

The values of K (Table 5) are on the whole fairly constant for the great majority of the salts compared. The variations from the mean of 2.2 are due almost entirely to the fact that mercury is not so poisonous as it should be, and that cobalt is a good deal less toxic than the theory demands, while nickel is a little more toxic. The explanation of these variations is no doubt to be found in part in the dissociation. I have assumed throughout that the dissociation is complete. This has been done for the sake of simplicity. It is, however, certain that the dissociation of mercury chloride even in these dilutions is far from

*Some modification or explanation is necessary of the conclusion of a former paper that oppositely charged ions must have of necessity an opposite action. This is in one sense true. That is, the positive ion in combination with the proteid, if the latter is electronegative, must constantly be neutralizing the negative charge and producing undissociated albumin. It may be stated in this sense that the positive ion always tends to precipitate an electronegative albumin. It happens, however, that the power of neutralizing the charges of the colloid—that is, of reducing ionization—varies greatly in different cations, being greatest in those of high ionic potential, and least in those of low. If, therefore, we have, as we do have in a protoplasmic system, colloids in a state of equilibrium with ions already present, the particular direction of the change in state of that equilibrium produced by the substitution of new positive or negative ions for those already present will depend on the relative potentials of the ions present and those introduced in their places. The actual effect observed, therefore, of replacing an ion of high potential with that of a low, will be the direct opposite of that produced by replacing the ion with an ion of still higher potential, and in this case there will appear to be an antitoxic or antagonistic action between two ions of the same character of charge. In an earlier discussion of this matter I neglected to take into account the great importance of the ions present in protoplasm. For example, suppose the ions in the protoplasmic system to be mainly sodium; and let us suppose that potassium has a lower potential than sodium, while calcium has a higher potential. If one substitute calcium for the sodium, the result will be to precipitate in part the electronegative colloids in the protoplasm. If, however, potassium be substituted for the sodium, the result will be to dissolve still further the colloids. In this case potassium and calcium will appear to exert an antagonistic action toward each other. If, however, the ions already in the protoplasm are of higher potential than calcium, then both potassium and calcium will produce the same kind of an action on the protoplasmic colloids. The results obtained by Loeb, Loeb and Giess, Miss Moore and myself on toxic and antitoxic action of salts thus have a very simple explanation.

complete. If we assume it to be only 50 per cent, it would bring the mercury into its proper position. Similarly with cadmium, which is a trifle too low in its toxicity, this dissociation is certainly incomplete. As regards cobaltous chloride, which is noticeably below what it should be, I have no explanation to offer except to point out that it occupies the same exceptional position toward some other forms of protoplasm. Possibly its power of forming double compounds with ammonia and its derivatives may have something to do with its anomalous behavior.

The constancy of K must be regarded, I think, with the exceptions just mentioned, as satisfactory, when it is remembered that the method of determining the minimum fatal dose—that is, by dilution—does not permit of very accurate figures.

C evaluated from these figures was— 4.111. (See Formula 4.)

TABLE 6.

RESULTS ON *Fundulus* EGGS.

Salts Compared	$(E_c'+E_a'-E_c''-E_a'')$	$\log \frac{V_1}{V_2}$	K
$CuCl_2-MnCl_2$	(1.403)	3.574	2.547
$HgCl_2-MnCl_2$	1.816	4.097	2.260
$HgCl_2-CuCl_2$	0.412	0.5229	1.269
$NiCl_2-MnCl_2$	0.848	2.0969	2.473
$CoCl_2-MnCl_2$	0.843	1.796	2.131
$HgCl_2-MgCl_2$	2.241	4.398	1.964
$CuCl_2-MgCl_2$	1.828	3.875	2.120
$CuCl_2-NiCl_2$	0.556	1.477	2.655
$HgCl_2-CoCl_2$	0.972	2.301	2.367

Mean value $K=2.20$

TABLE 7.

COMPARISON OF ZN WITH ALL OTHER METALS.

Salts Compared	$(E_c'+E_a'-E_c''-E_a'')$	$\log \frac{V_1}{V_2}$	K
$ZnCl_2-MgCl_2$	0.726	2.602	3.581
$ZnCl_2-MnCl_2$	0.302	2.301	7.610
$CdCl_2-ZnCl_2$	0.345	1.194	3.461
$CoCl_2-ZnCl_2$	0.541	−0.5051	−0.934
$NiCl_2-ZnCl_2$	0.546	−0.2041	−0.374
$PbCl_2-ZnCl_2$	0.613	0.795	1.296
$CuCl_2-ZnCl_2$	1.102	1.273	1.182
$HgCl_2-ZnCl_2$	1.514	1.7959	1.186
$AgNO_3-ZnCl_2$	1.597	2.097	1.314

Mean K 2.03

The results obtained upon *Fundulus* (Tables 6 and 7) do not give quite such constant values as those of McGuigan, and indeed with so complex a system this was not to be expected. However, the

average value of K in these results is almost exactly the same as that of McGuigan; i. e., about 2.2. The exceptions in these results are, with the expection of cadmium, the same as those recorded by McGuigan, cobalt being too little toxic and zinc too toxic. I have determined K for zinc chloride in comparison with all other metal chlorides, expecting that, while the ratio would be too low for all other metals above it in the scale of ionic potentials, it would be too high for all metals below it, and these two errors should neutralize each other, provided the metals were about equally distributed above and below zinc. The result (Table 7) gave for the mean K 2.03, which is a litttle low, but fairly close to the mean 2.2 already obtained.

I have also determined (Table 8) the fatal dose just sufficient to stop swimming in two minutes in rapidly swimming cultures of *Volvox globator*. Four metals were investigated—i. e., silver, cadmium, manganese, and magnesium. K was assumed to be 2.2, the same as that for *Fundulus* and diastase, and the constant C was calculated. The result was as follows:

TABLE 8.

Salt	log V	C
$AgNO_3$	5.477	−3.5
$CdCl_2$	2.699	−3.6
$MnCl_2$	1.699	−3.11
$MgCl_2$	0.699	−3.13
		Mean−3.33

The constant C is thus found as constant, as could be expected from the methods used and the variability of the cultures.

Undoubtedly the most consistent and accurate results thus far obtained are those of McGuigan upon the minimum fatal dose of salts for the diastatic ferment. These results are plotted in Fig. 3. I have used only those results which were obtained with the metals Mg, Mn, Zn, Cd, Co, Ni, H, Cu, Hg, and Ag, for the reason that the solution tension, and hence the ionic potential, of all metals above magnesium—i. e., Ca, Sr, Ba, Na, K, Li, and Cs—are still so very uncertain as to make the comparison of ionic potential and fatal doses of little value. In Fig. 3 the line A B represents the formula.

$$\log V = C + K(E_{\text{cation salt}} - E_{\text{cation colloid}}) + (E_{\text{anion salt}} - E_{\text{anion colloid}}) .$$

The ordinates represent the logarithms (common) of the dilution (V) of the minimum fatal dose; the abscissæ represent the differences between the ionic potential of the cation of the colloid, which is assumed to be potassium at 2.9 volts and the ionic potential of the various

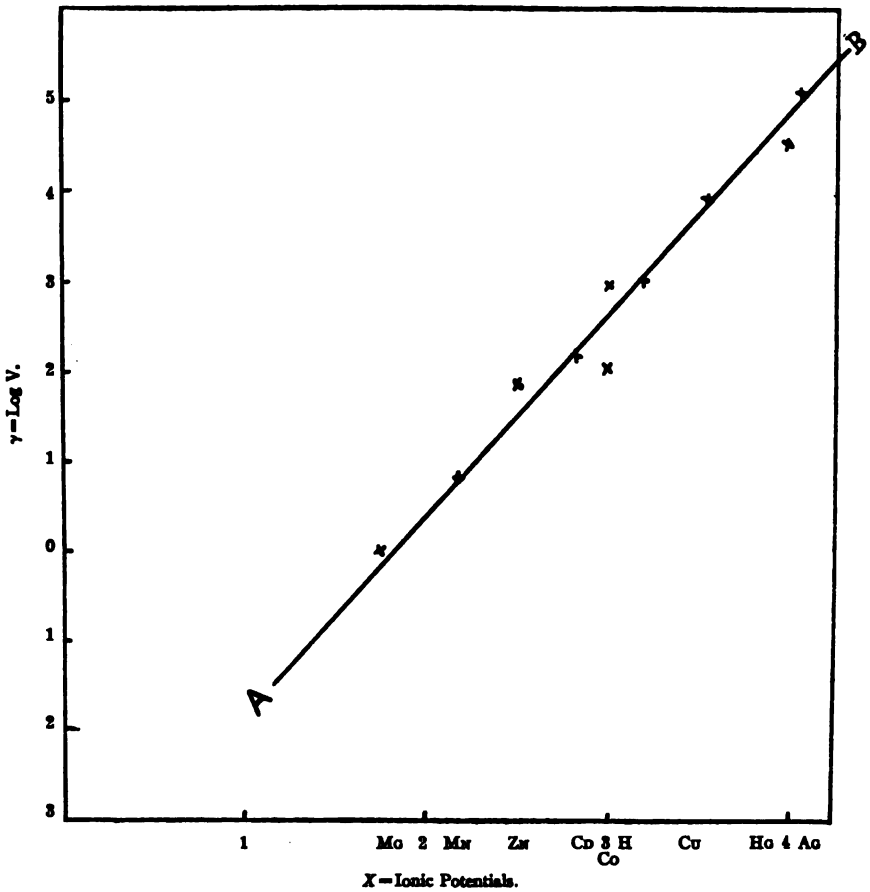

FIG. 3. Plat of results obtained with diastase. Ordinates are the logarithms of the dilution of the minimum fatal dose. Abscissæ represent the difference between the ionic potentials of K. Cl and potentials of ions of toxic salts.

metals. The line makes an angle with the X-axis, the tangent of which is 2.23, and it cuts the Y-axis at -4.11 (or C).

The remarkable closeness with which the various metals approximate to this line will be apparent.

If now we turn to my results on *Fundulus* (Fig. 4), two things are at once clear from an inspection of Table 6 and Fig. 4. The first result is that the gradient or slope of the line which represents the relation between toxicity for *Fundulus* eggs and the ionic potential is

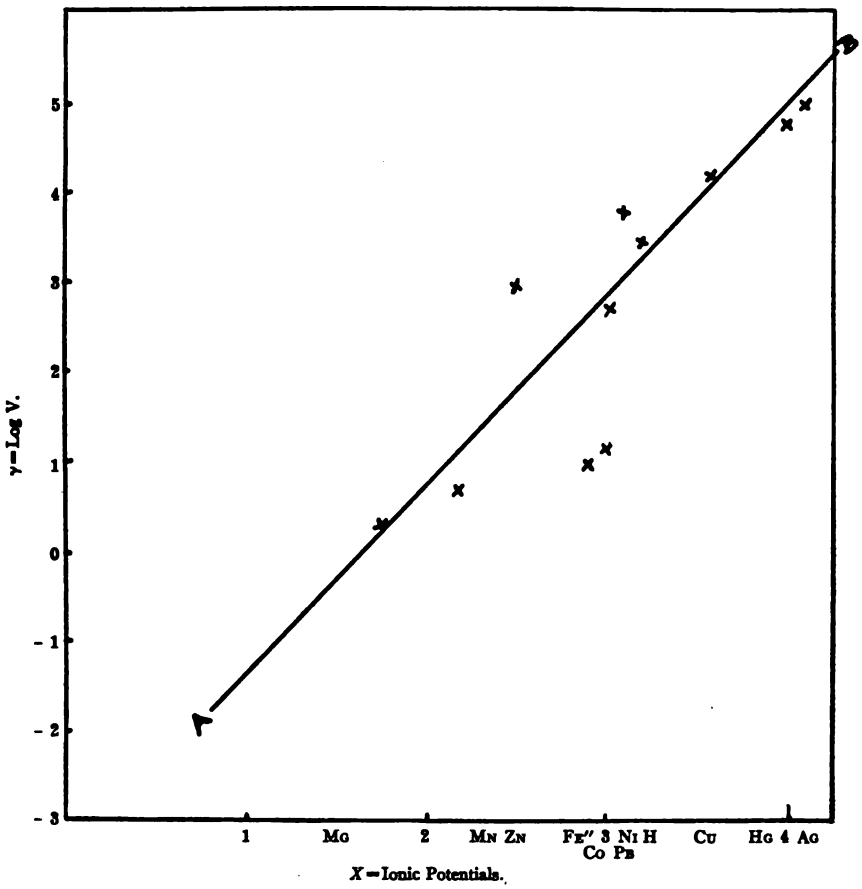

FIG. 4. Plot of results obtained with eggs of *Fundulus heteroclitus*. Ordinates and abscissæ as in 3.

exactly the same as that found for diastase. This must be regarded as confirmatory evidence of some value of the probable correctness of our attempt to work out a numerical relationship between toxicity and ionic potential. The second result which is very clear is that there is in the case of *Fundulus* greater variations than in

the case of the ferment. This is, of course, to be expected since in the egg we are dealing with a vastly more complicated system than in the ferment. We have not only a variety of ferments and substances which might be differently affected by the salts, but the eggs are in addition separated from the water in which the salts are by membranes which are known to be variously permeable to different salts. The fact that the results show so good an agreement with the computed values is hence the more satisfactory. As regards the exceptions, it will be observed by comparing Figs. 3 and 4 that they are in general the same in each, only the deviations are greater for the egg. Thus zinc, which toward diastase was somewhat more poisonous than it should be, toward the eggs is very much more toxic. Cobalt, which is too little toxic toward diastase, shows the same relationship toward the eggs; the same is true of mercury. On the other hand, certain very interesting special exceptions occur. Thus cadmium, which toward diastase occupies almost exactly its theoretical position, is toward *Fundulus heteroclitus* eggs extremely toxic. It is, in fact, so toxic and so far out as to show that there is some specific and special reason for its aberrance. I have accordingly disregarded it. On the other hand, lead, which was for some special reason far out of place toward diastase, is here almost where it should be.

As regards the toxicity of the metals sodium, potassium, and lithium it will be noticed that they are relatively more toxic toward *Fundulus* than toward the diastase. The reason for this may possibly be that the strong solutions are in themselves harmful by their osmotic action on the cells.

I have also incorporated the results of a few observations made upon the rapidly swimming culture of *Volvox globator* (Fig. 5). I determined the concentration just sufficient to stop swimming within two minutes. The computation of the constant a, from the results gives a very satisfactory agreement.

e) *Other results on toxicity.*—The results of Caldwell on bromelin, the proteolytic ferment of the pineapple, I have been unable to bring to any satisfactory numerical agreement. But while Caldwell's results cannot be brought into quantitative relationship with McGuigan's and mine, the general trend of the results is plainly the same. The order of the toxicity of the metals is in nearly all cases as it should theoreti-

cally be, so far as he has tried those of which the solution tension is known. The same exceptions are also apparent. Thus cobalt is not sufficiently toxic, and zinc is too toxic for the rule. Lead is about where it belongs, but in this case barium is the marked and peculiar

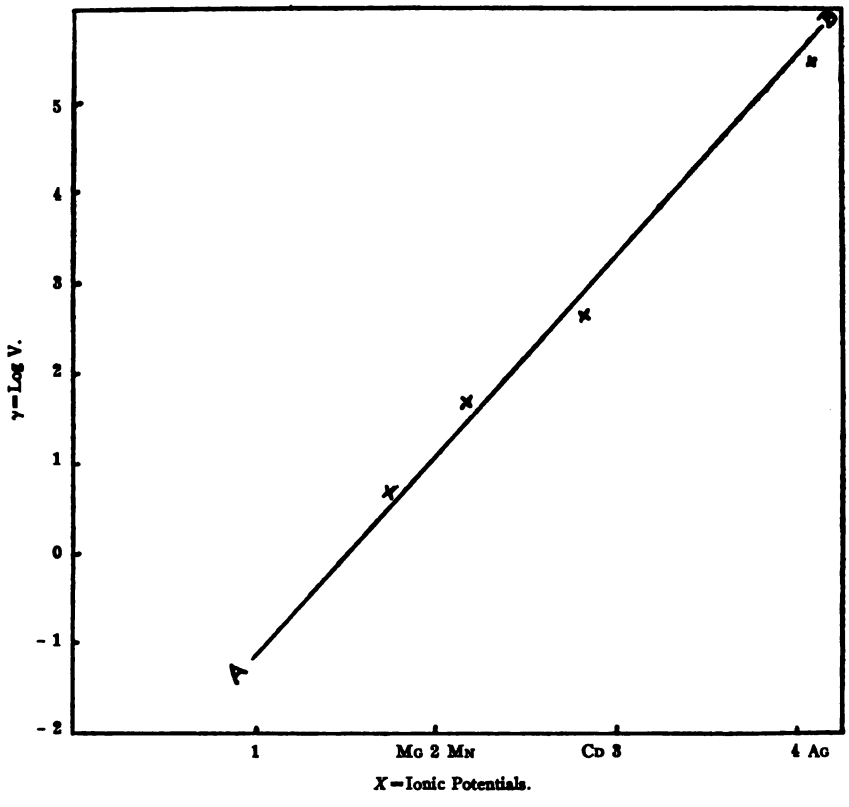

FIG. 5. Plot of results obtained with *Volvox*. Abscissæ and ordinates as in Fig. 3.

exception, in place of the lead toward diastase, and cadmium toward the *Fundulus* egg.

The results of Heald (Table 1) so far they have been obtained with the salts we are examining, show much the same order of efficiency. Thus for *Pisum sativum* the order of toxicity is: Ag, Hg, Cu, Ni, Co, and H; while for *Zea mais* it is Ag, Cu, Hg, Ni, Co, and H. These results are not of a sufficiently definite character, based as they are on the growth of roots, to enable very accurate quan-

titative comparisons. It will be noticed that nickel is more poisonous than cobalt for *Pisum sativum*, and far more poisonous than cobalt for *Zea mais*. Both of these roots appear less sensitive to acids than to the metals, the hydrogen ion being less toxic than nickel. With *Lupinus*, while the general order is the same as that observed elsewhere, cadmium is here more poisonous than copper—just the exception noted for *Fundulus*.

The results of Clark and Stevens upon mold spores are also for the purposes of quantitative comparisons unsatisfactory. These spores appear to be surrounded by such membranes, or to have so great a resistance, as to make the interpretation of results very doubtful, although there can be no doubt that the general trend of the results is the same as that already noted.

True and Kahlenberg's results (Table 1) are more satisfactory, but, owing to the fact that these authors did not study many of the metals, and also did not pretend to fix the fatal point with accuracy, their results are not satisfactory for quantitative treatment. True's results upon the toxicity of the salts of the various acids are unfortunately unavailable for our purposes, owing to the uncertainty of the ionic potential of these anions.

f) The solubility of globulin in salt solutions.—In a previous paper[1] I showed that the solubility of sodium albuminate (egg albumin in alkaline solution) in different salt solutions was determined by the tension-coefficient of the salt. By the tension-coefficient was meant the difference between the solution tensions of the ions divided by their sum. The numerator of this fraction should be the ionic potentials instead of the solution tensions. I showed that in an alkaline solution the solubility was greater in sodium iodide than in the bromide or chloride, and that when the ionic potential of the cation surpassed a certain figure the salts precipitated the albumin. Table II[3] shows the relationship (qualitative) between the ionic potentials of salts and their power of dissolving or precipitating such albumin, both the unboiled and the boiled. It will be seen by an inspection of the table that the ionic potential arranges the salts in the order of their action on the albumin.

Osborne and Harris[2] have estimated quantitatively the solvent

[1] MATHEWS, *Amer. Jour. Physiol.*, 1905, 14, p. 204.
[2] OSBORNE AND HARRIS, *ibid.*, 1905, p. 151.
[3] MATHEWS, *loc. cit.*, p. 211.

power of many salts for the globulin of the hemp seed edestin. I append their results here to show how far they agrce with the theoretical deductions which were worked out on p. 102. In Table 9 $E_c - E_a$ represents the difference between the ionic potentials of the salts;

TABLE 9.

Salt	$E_c - E_a$	c.c. to Dissolve 1 gr.	K
KI	−2.123	5.7	
KBr	−1.65	10.	.51
KCl	−1.226	15.1	.44
NaI	−1.743	5.7	
NaBr	−1.27	9.3	.44
NaCl	−0.846	12.8	.33
LiBr	−1.10	12.1	
LiCl	−0.646	19.1	.44

the third column represents the number of c.c. of a normal solution of the salts which will just dissolve one gram of edestin. I have taken these figures from the chart given by Osborne and Harris. They are only approximate. The theory requires that the more negative the difference $E_c - E_a$, the smaller the amount of salt necessary to dissolve a given amount of the edestin. It will be seen that this relationship holds very well if we compare the iodides, chlorides, and bromides of sodium, lithium, and potassium respectively. Unfortunately, the great uncertainty of the solution tensions and ionic potentials of sodium, potassium, and lithium, prevent quantitative comparisons between the different metals. In the fourth column under K, I have computed the constant by the formula on p. 102, using instead of the logarithm of the dissolution the logarithm of the ratios of number of c.c. of the different solutions necessary to dissolve one gram. The figures are placed between the salts compared. The formula used was:

$$\frac{1}{(E_{c_1} - E_{a_1}) - (E_{c_2} - E_{a_2})} \log \frac{\text{c.c.}_1}{\text{c.c.}_2} = K \ .$$

Osborne and Harris draw the conclusion that the solubility is independent of the nature of the base, but I think their figures speak for themselves. The differences between potassium, lithium, and sodium are small, to be sure, but nevertheless apparent. The importance of the base becomes obvious so soon as any other salts are examined in which the base has a higher ionic potential than

these. Then it is seen that whether the salt dissolves the edestin or converts it into a curdy mass depends mainly on the base. The reason why so slight differences exist in the solvent power of the cations, sodium, potassium, lithium, barium, calcium, and magnesium, is shown by an examination of their ionic potentials, which are very low and probably about the same in each. The solvent power of manganese and ferrous chlorides is low.

Cu, Cd, Cr, Co, Fe‴, Pb, Hg, Cu, Al, Zn chlorides and nitrates all fail to dissolve.

The solvent powers of the anions arrange themselves in descending order as follows: CrO_4, SO_3, S_2O_3, I, Br, Cl, SO_4, which is almost certainly the descending order of the ionic potential of these ions. The fact that these bivalent ions of high ionic potential dissolve, instead of precipitating, the colloid, and that the valence of the anion is unimportant, is, in my opinion, good evidence that the edestin in such solutions is electronegative and not electropositive; for, as has been shown by Hardy and many others, the valence of the ion of the same sign as the colloid is immaterial, but toward a colloid of opposite sign it is very important.

The peculiarity of the dissolving action of the heavy metal acetates[1] also receives a possible explanation. In these solutions which dissociate acetic acid the edestin becomes electropositive. Consequently it is no longer precipitated by the positive ions, but receives energy from them, and is rendered more positive, and hence more soluble, than it was before. The cause of the failure of the chlorides to dissolve the edestin in acid solution would be that given by Osborne, that in such solutions, where there are many hydrogen ions, it is changed into edestan as an insoluble product.

I append here also a summary of the results of Pauli[2] illustrating the same facts, showing the parallelism between the solvent or precipitating power of the anion upon albumin and its potential energy content. The parallelism is certainly unmistakable.

Order of precipitation	$SCN > I > Br > NO_3 > Cl > C_2H_3O_2$
Ionic potential	83(?) − .79 − 1.27 − 1.694

g) The phenomena of stimulation of cells.—I found that the salts ranged themselves very simply in the order of their energy content,

[1] OSBORNE AND HARRIS, *loc. cit.*, p. 165.

[2] PAULI, *Hofmeister's Beiträge*, 1905, 6, p. 249.

so far as their action, stimulation or depression, on the motor nerve was concerned. But it is clear from what has been said, that no such simple arrangement is to be anticipated in studying a complex system such as a cell undergoing rapid change. In such a system all we observe from the action of the salt is a definite result, which implies a certain change in the system. This result may be a stimulation, such as a muscle contraction, or nerve impulse. Evidently the same result may be brought about in several different ways: either by direct action of the salt on the particular part of the system which undergoes change; or, indirectly, by the salt altering another part of the system, so as to produce or check the result. It is conceivable that the same salt may have a double action: by one action tending to produce the direct change of the response; and indirectly by action on another part of the system having as a result the setting-up a process which will check its own direct action.

Something of this last process obtains, I believe, in protoplasm generally, so that strict adherence to the law of ionic potential action is not to be expected; but a general adherence is to be expected, and the facts show it exists.

An interesting example of this stimulating action is seen in the extrusion of polar globules in *Chaetopterus* eggs. These eggs undergo the first processes of maturation before they are shed, but they do not extrude the polar globules until fertilized. The first polar spindle is formed and comes to rest in the equatorial plate-stage. Evidently in this egg there is not sufficient energy in the spindle to overcome the resistance offered by the surface tension or

TABLE 10.

MINIMUM CONCENTRATION OF VARIOUS SALTS CAUSING EXTRUSION OF POLAR GLOBULES IN CHAETOPTERUS.

Salt	Concentration
Na_3 citrate	$\frac{7}{108}$ *n*
Na_2SO_4	$\frac{1}{8}$ *n*
KI	$< \frac{1}{48}$ *n*
KBr	$< \frac{1}{40}$ *n*
KCl	$\frac{1}{18}$ *n*
NaCl	$\frac{5}{88}$ *n*
LiCl	$< \frac{1}{8}$ *n*
$CaCl_2$	$\frac{1}{18}$ *n*
$MgCl_2$	0(?)
$MnCl_2$	0
$CdCl_2$	$< \frac{1}{1,600}$ *n*
$CuCl_2$	$< \frac{1}{24,500}$ *n*

other factors, of the egg. Salts may cause extrusion either by increasing the energy in the egg, or by decreasing the surface tension.

Unfortunately the end-points were not determined accurately in many instances, but the figures (Table 10) suffice to show that the stimulation of this particular function was greatest in copper and cadmium, fell to nothing in manganese, was doubtful in magnesium, and then increased as salts having anions of higher ionic potential were used. In other words, if we begin with manganese or magnesium chlorides and pass to salts having a higher potential energy of either the anion or cation, this function is stimulated, and less of the salt must be used as the energy content increases.

GENERAL CONCLUSONS.

The general conclusions of this investigation are:

1. The action of salts upon the protoplasmic system is due chiefly to the ions of the salt.

2. The particular result obtained—toxic, stimulation, or depression excited by any salt solution—is caused, in part at least, by the substitution of the ions in the protoplasmic system, and in large measure in combination with the protoplasmic colloids, by the ions of the salt solution used.

3. This substitution causes a disturbance of the equilibrium of the protoplasmic system, which, if sufficiently pronounced, leads to destruction.

4. *The power of different ions to upset the ordinary state of the protoplasmic system depends on the difference between the potential energy content of the ion which is replaced, and that which is introduced. This difference in potential energy content is determined by the difference in the intensity factor of the potential energy of the ions—i. e., by differences in the ionic potentials.*

5. It follows from 4 that the ions must arrange themselves in toxic power according to their available potential energy contents (ionic potentials). This was shown to be the case not only for toxic action, but also for stimulating and depressing action.

6. It was shown that a good numerical relation exists between the *available* potential energy of any salt and its minimum fatal dose, so that for simple systems the minimum fatal dose can be very

closely calculated from the available potential energy of the salt, if certain constants are known.

7. A method was found for computing the ionic potential of various ions from the solution tensions.

8. In the case of *Fundulus* eggs, which were particularly investigated from this point of view, the order is also consonant with the theory, but the quantitative relationships, while fairly good, are not so uniform as in the case of diastase.

The character of the action of any given salt solution on protoplasm must of necessity depend upon the character of the ions, metal and metalloid, already in combination with the protoplasm. This results as a necessary consequence of the theory, and it explains the fact that toward different cells, and toward the same cell after exposure to different salt solutions, the same salt solution may exert a different action, being at times stimulating, at other times depressing.

It has been shown that the physiological action of any salt solution is a function of the *available potential energy of its ions*. The action of the organic drugs will, I think, also be found to depend, in part at least, on the available potential energy of the dissociated particles of the drug.

AN INSTANCE OF THE APPARENT ANTITOXIC ACTION OF SALTS.*

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(From the Physiological Laboratory of the Massachusetts Institute of Technology and the Biological Department of Ripon College.)

THE idea that certain salts may neutralize toxic properties of other salts so that a suitably proportioned mixture may be harmless to living protoplasm though its constituents are individually harmful, is one which we owe especially to Loeb[1] and Mathews.[2] There is much evidence favoring such a conception. The observations of Loeb upon the abnormal behavior of skeletal muscles in sodium-chloride solutions and upon the failure of fish embryos to develop in solutions of this single salt are likely to be ranked as classic. He found that the muscles no longer twitched when certain salts, notably those of calcium and potassium, had been added to the bath, and that the embryos prospered when similar corrective additions to the medium had been made.

These instances no longer stand alone, for they have been repeatedly paralleled;[3] but the cases first described remain fair types of the class. Whether we do well to use the terms "toxic" and "antitoxic" in this connection may be questioned, and depends upon what theory we adopt to account for the facts. The terminology has been criticised by Howell,[4] and recently by Osborne.[5] When it is said that a certain salt by itself is toxic, the meaning seems often to be merely that it is *insufficient*, and to say that a second salt is antitoxic to the first implies little more than the thought that it supplies some need of the tissue not met by the other. But we may keep the picturesque and convenient expressions without being dogmatic as regards their application.

In a recent paper[6] the authors dealt with the action of lithium upon skeletal muscle. The experiments recorded at that time gave ground for the belief that lithium-chloride solutions are among the least harmful to which the muscle may be exposed, and that such solutions preserve the tissue very nearly as well as the usual

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"physiological" mixtures. At the conclusion of that paper we questioned whether it might not be possible to find a mixture of lithium chloride with some other salt which should still more closely approach the normal fluid in its conserving properties. When we expressed this hope, we had already made some tentative experiments with suggestive results. These we have since repeated and extended, and our observations may now be outlined.

Some time ago we investigated the duration of irritability and the working capacity of muscles immersed in solutions of magnesium chloride. We found that the loss of excitability is rapid, and that two or three hours in such a bath makes the gastrocnemius unresponsive to the strongest stimulation. Thus solutions of magnesium chloride alone are distinctly unfavorable to the muscle protoplasm. However, we were led to test mixtures of the chlorides of lithium and magnesium (with the former in large excess), and to compare the records written by muscles treated with these mixtures with those obtained from companion muscles in lithium chloride alone. It seemed not unlikely that these combinations might prove somewhat superior to the straight lithium chloride, as well as vastly better than the unmixed magnesium chloride. In the current phraseology we looked for an antitoxic action on the part of magnesium.

The specific influence of magnesium salts upon various living tissues has not been sufficiently investigated. At the time of this writing* there has appeared the first of a promised series of papers by Meltzer and Auer,[7] which will doubtless make much new and exact information available. As these writers point out, the conspicuous property of magnesium salts in large quantity is a depressing or paralyzing one, and they suggest that the physiological rôle of these salts, which are somewhat abundant in animal cells and fluids, is inhibitory. If this is the case, there is a closer relationship between magnesium and potassium than between magnesium and calcium. It should be easy to define the comparative effects and the antagonisms of these salts by experiments with plain and cardiac muscle, and we have undertaken work along these lines.

In the present contribution we shall confine ourselves to the

*November, 1905.

effect upon skeletal muscle of various mixtures in which the chlorides of lithium and magnesium were the principal ingredients.*

When a muscle is exposed to a bath, there are at least three ways in which we may estimate the effects of the immersion. First, we may simply find out the duration of its irritability, noting when in the course of hours or days it ceases to respond to strong stimuli; in this case we should apply shocks only occasionally, and the muscle would not have performed much work. Second, we may exhaust the muscle by severe and repeated stimulation after a longer or shorter period in the bath, and thus obtain a rough measure of its working capacity. For this purpose a work-adder may be used, or the area of the record traced on a slowly moving surface may be noted. Third, we may determine the irritability of the immersed muscle from time to time by varying the strength of the stimuli and recording the position of the secondary coil corresponding to maximal and minimal responses. We kept all three points in view, but placed emphasis upon the second—the total performance of the muscles when stimulated strongly and frequently. Sometimes a thousand contractions went to form a record.

All our experiments were comparative. We used companion muscles from the right and left legs of frogs, so that we might have controls which at the outset should be practically identical with their fellows in potential energy and irritability. The muscles worked upon equal levers similarly adjusted and weighted. The stimulating current passed through both preparations, traversing each lengthwise and being led from one to the other by a flexible bit of metallic tinsel. In most of the experiments the shocks were derived from a cog-wheel interrupter driven by clock-work. Break-shocks alone were brought to bear on the tissue, the makes being eliminated by a mechanical device. The stimuli were applied from 30 to 60 times per minute. Usually the stimulation was supra-maximal, and the muscles were out of the solutions when working, so that there could be no scattering of the current. They were frequently washed during their prolonged series of contraction The solutions were made from salts of high purity and tested as

*Sometimes the chlorides of calcium and potassium were also present in the small percentages of a Ringer's mixture. Their presence did not affect the general nature of the results, and it is needless to dwell upon the minor modifications which they probably brought about.

to the freezing-point. By this means we were able to use media which had a uniform osmotic pressure appropriate to the frog's tissues. The value Δ varied little from 0.50°.

About thirty experiments of a reasonably consonant character yield data on which the following conclusions are based. The order and duration of the different trials were purposely contrasted as far as possible. Sometimes the immersions were measured in minutes, sometimes in days. When low temperatures were maintained, it was found possible to keep preparations for nearly a week in extreme instances.

Skeletal muscles are better preserved by a proper mixture of lithium and magnesium chlorides than by lithium chloride alone. It is naturally difficult to fix upon the optimum ratio between the two chlorides. Seven parts by volume of lithium-chloride solution to one part of magnesium chloride is a highly favorable proportion. If we increase the magnesium much above this fraction, it shows its influence in lowering the irritability of the muscle.

The mixtures favor a maximal performance of work by muscles whether the exposure has been short or long. This is apparently not true unless the stimulation is amply strong, for the irritability of the muscle is probably somewhat depressed in the presence of even a small quantity of magnesium salts. Closely connected with this is the fact that the earlier contractions of the muscle in straight lithium chloride may exceed those of the companion in lithium-magnesium mixture, yet the latter outdoes the former because it outlasts it. This seems to us a very significant fact; perhaps we have here the key to the beneficial working of magnesium. It may be conceived to have the action of economizing the metabolism, of preventing a wasteful expenditure of substance and energy in the muscle-cells. We have noticed that it lessens contracture in fatigued muscles. This is thoroughly in harmony with the broad statements of Meltzer and Auer, whose work was not known to us when we were first led to these opinions.

We believe, then, that magnesium in small quantities is a conserving element in contractile tissue. We may again question whether it is best to designate this action as antitoxic, though there is a marked similarity between our experiments and those upon

eggs and larvæ, in which the antagonism of ions has been made apparent. It is not necessary to assume that there is any peculiar power of mutual neutralization in lithium and magnesium. We may reasonably suppose that the plain lithium-chloride bath is not of the most favorable nature, because the metabolism of the muscle immersed in it is not of the most economical character. An admixture of magnesium chloride secures economy and a better direction of the energy set free. But an excess of magnesium chloride is naturally unfavorable, because the same action carried farther must result in depressed excitability.

It seems to us a legitimate expectation that many other cases in which mixtures of two salts have proved superior to either by itself may be explained in equally simple ways.

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EXPERIMENTS WITH BACTERIAL ENZYMES.*

EDWIN O. JORDAN.

INTRODUCTORY.
TECHNIQUE.
CONDITIONS OF GELATINASE FORMATION.
Composition of Medium.
Reaction of Medium.
PHYSICAL CHARACTERS.
Resistance of Gelatinase to Heat.
Filtration.
CONDITIONS OF ACTIVITY.
Reaction of Medium.
Temperature.
THE ACTION OF FORMALIN UPON LIQUEFIED GELATIN.
THE RELATION BETWEEN BACTERIAL GELATINASES AND BACTERIAL HEMOLYSINS.
SUMMARY AND CONCLUSIONS.

INTRODUCTORY.

IT was first shown in 1886 by Bitter[1] that the liquefaction of gelatin by bacteria depends upon enzyme action. Rietsch,[2] Senger,[3] Jerosch,[4] Sternberg,[5] Krabbe,[6] and others confirmed and somewhat extended Bitter's observations, and in 1890–92 Fermi[7] published comprehensive investigations upon the properties of gelatin-liquefying and other bacterial enzymes.

The power of bacterial filtrates to liquefy gelatin is a more or less independent quality. It is true that various experimenters have not distinguished between the gelatin-liquefying ferments and other "proteolytic" enzymes, and have openly or tacitly assumed that gelatinolytic ability is a measure of general proteolytic power. Malfitano,[8] however, has shown that the albuminolytic and gelatinolytic properties of anthrax filtrates are really distinct and should not be confounded. This writer states that it is possible to weaken or even destroy the albuminolytic power of an anthrax filtrate without

* Received for publication January 2, 1906.

[1] *Arch. f. Hyg.*, 1886, 5, p. 245.
[2] *Jour. de pharm. et de chimie*, 1887, 16, p. 8.
[3] *Baumgarten's Jahrb.*, 1887, 3, p. 104.
[4] *Baumgarten's Jahrb.*, 1887, 3, p. 104.
[5] *Ibid*, p. 363.
[6] *Jahrb. wiss. Bot.*, 1890, 21, p. 520.
[7] *Centralbl. f. Bakt.*, 1890, 7, p. 469; *ibid.*, 1891, 10, p. 401; *ibid.*, 1892, 12, p. 713.
[8] *Compt. rend. de la Soc. de Biol.*, 1903, 55, p. 843.

materially attenuating its gelatinolytic power. As a matter of fact, Rietsch[1] in 1887 called attention to the existence of at least two different proteolytic ferments, and Fermi[2] in 1891 pointed out that the action of the bacterial ferments upon fibrin and egg albumin was relatively feeble and was not correlated with their action upon gelatin.[3] Pollak has recently stated that serum digestion and gelatin digestion by trypsin solutions depend upon the action of two different ferments. There is reason, therefore, for considering the gelatinases[4] as a definite class of bacterial proteases. According to Mavrojannis,[5] two kinds of gelatinases occur: (1) those that decompose gelatin with the formation of gelatoses, and (2) those that push decomposition as far as the formation of gelatin peptones and perhaps beyond. As will be shown presently, however, it is doubtful if this distinction can be maintained.

TECHNIQUE.

In the work here described, a uniform procedure has been followed in testing the gelatinolytic action of bacterial filtrates and cultures. Five c.c. of neutral carbol gelatin (8.0 per cent gelatin; 0.25 per cent phenol) has been used as the standard quantity to be subjected to enzymic action; a measured quantity of the enzyme-containing fluid is added to this while the gelatin is warm (35°–40°); the control tubes are diluted with 0.85 per cent NaCl solution correspondingly; enzyme and gelatin are thoroughly mixed by shaking, and incubation at 36°–37° for 20 hours follows. The tubes are then cooled in ice-water for 15 minutes, after which they are removed and allowed to stand at room temperature for 15 minutes, when the extent of solidification is recorded. In case the action of the enzyme is complete, the contents of each tube remain entirely liquid. I have termed the smallest amount necessary to produce complete liquefaction under the conditions specified the minimum lytic dose. If the action is only partial the lower half or two-thirds of the gelatin may solidify, or the whole mass may be semi-solid. Control tubes incubated for a similar period show complete solidification without a trace of tremor on

[1] *Loc. cit.*, p. 11.
[2] *Centralbl. f. Bakt.*, 1891, 10, p. 401.
[3] *Beiträge z. chem. Physiol. u. Pathol.*, 1904, 6, p. 95.
[4] Pollak uses the term "glutinases."
[5] *Ztschr. f. Hyg.*, 1903, 45, p. 108; *Compt. rend. de la Soc. de Biol.* 1903, 55, p. 1605.

shaking.* The reactions given for the nutrient media are based on the phenolphthalein neutral point.

CONDITIONS OF GELATINASE FORMATION.

Composition of medium.—One inquiry of obvious theoretical interest is whether gelatinase is formed more abundantly in gelatin-containing media than in media in which no gelatin is present. Lauder Brunton and Macfadyen,[1] as the result of their studies, concluded that "an enzyme is formed in meat broth which liquefies gelatin, and does so more surely and quickly than the enzyme formed in gelatin itself." Fermi,[2] on the other hand, found that in general the enzyme production in broth was less abundant than in nutrient gelatin. Recently Abbott and Gildersleeve[3] have stated that in their observations "the enzyme content of completely liquefied gelatin cultures was always marked, and was in general more marked for all species than was the case with any of the other culture media used." These authors attach importance to this finding as tending to support the doctrine of the over-production by the cell of particular receptive atom groups. In this sense the presence of gelatin in the culture medium would be expected to cause a more active elaboration of gelatinase. The influence of gelatin is hence regarded by Abbott and Gildersleeve as "stimulating bacteria to the active production of liquefying enzymes."

My results do not show that gelatinase production in nutrient gelatin always outstrips or exceeds the production in nutrient broth. The following table illustrates the conditions observed in a series of tests.

The table shows: (1) that some cultures develop gelatinase more quickly and abundantly in nutrient broth than in nutrient gelatin prepared from the same lot of broth; (2) that other organisms produce more gelatinase in nutrient gelatin than in nutrient broth; (3) that in other cases there is little difference. In one instance (*B.*

* Somewhat similar methods have been employed by Fermi, Malfitano and others. Fermi (*Arch. f. Hyg.*, 1906, 55, p. 140) has recently expressed a preference for the use of solid gelatin in place of fluid gelatin as a means of testing the action of these enzymes, but the disadvantages of his method seem to me greater than those of the one here described. I have not found it difficult to obtain comparable results when attention is paid to details of mixing, temperature, etc. Some of the experiments I have made could hardly be carried out by the use of solid gelatin.

[1] *Proc. Roy. Soc.*, 1889, 46, p. 542.

[2] *Centralbl. f. Bakt.*, 1891, 10, p. 401.

[3] *Jour. Med. Res.*, 1903, 10, p. 42.

TABLE I.

COMPARISON OF GELATIN AND BROTH CULTURES.*

Organism	Grown at	Medium	Minimum Lytic Dose in c.c. at the End of												
			2 Da.	3 Da.	4 Da.	6 Da.	8 Da.	10 Da.	12 Da.	16 Da.	22 Da.	26 Da.	39 Da.	59 Da.	8 Mos.
B. pyocyan.....	37.5°	Broth		0.5		0.03	0.01	0.01	0.01		0.03				0
		Gelatin		0.5		0.1	0.03	0.03	0.03						0.05
B. pyocyan.....	37.5°	Broth	0.3		0.1	0.03		0.01	0.005			0.03		0.03	
		Gelatin	0.3		0.1	0.01		0.01	0.01			0.01		0.03	
B. subtilis.....	37.5°	Broth	0	0.5	0.1	0.1			0.1	0.1	0.2				
		Gelatin	0	0	0.5	0.5			0.5	0.5	0				
" "	20°	Broth	0	0	0	0.5	0.5	0.5		0.3	0.3		0.3		
		Gelatin	0	0	0	0.3	0.3	0.3		0.1	0.1		0.1		
B. subtilis.....	37.5°	Broth			0.3		0.3		0.3		0.3				
		Gelatin			0.3		0.1		0.1		0.1				
" "	20°	Broth			0		0.5			0.5		0.5			
		Gelatin			0.1		0.05			0.05		0.05			
B. prodig.....	37.5°	Broth		0.5		0.3		0.3		0.3	0.3				
		Gelatin		0		0.3		0.1		0.03	0.03				
" "	20°	†Broth		0		0.5		0.05		0.05	0.01	0.005	0.01		
		Gelatin		0.5		0.1		0.1		0.05	0.03	0.03	0.03		
B. amylorub....	37.5°	Broth			0		0		0		0				
		Gelatin			0		0.8		0.8		0.8				
" "	20°	Broth			0.05	0.03	0.03	0.03	0.03	0.03	0.05		0.1	0.2	
		Gelatin			0.1	0.1	0.05	0.03	0.008	0.008	0.008		0.01	0.05	
B. prot.....	37.5°	Broth	0	0.3			0			0	0				
		Gelatin	0	0			0.3			0.3	0.3				
" "	20°	Broth		0				0		0.5	0.5				
		Gelatin		0				0.05		0.03	0.03				
B. fluor. liq.....	20°	Broth	0.1		0.03		0.01		0.01		0.01				
		Gelatin	0.5		0.1		0.1		0.1		0.1				
Sp. Finkl.-Prior.	20°	Broth			0.3		0.2	0.1	0.03		0.01	0.01			
		Gelatin			0.3		0.1	0.05	0.05		0.05	0.05			

* A negative reaction, indicated by O, signifies that 0.5 c.c. of the culture or filtrate is not able to effect complete digestion of 5 c.c. carbol gelatin in 20 hrs. at 37.5° C. Greater quantities than 0.5 c.c. were not used in these tests.

† At 20° there is only the small amount of liquefied gelatin to collect from, while at 37.5° the whole of the medium is liquid and the enzyme consequently diluted.

subtilis) cultures incubated at 37.5° gave more abundant production of gelatinase in broth than in gelatin, while cultures kept at 20° showed just the reverse. *B. subtilis* cultures in broth at 37.5° developed the enzyme earlier than those at 20°, while *B. proteus* cultures gave a larger amount of enzyme at 20° than at 37.5°. It is plainly incorrect to compare the enzyme content of the liquefied gelatin produced at room temperature with that of a whole flask of gelatin incubated at 37°. On the same ground, a comparison of broth and gelatin cultures at 20° is open to objection. Sometimes old gelatin cultures are considerably more potent than the corresponding broth cultures, but this is not invariably true. (Table I, *B. pyocyaneus*, 59 days; *B. prodigiosus*, 39 days.) This seems in some cases to be due to the loss of the original strength of the broth culture (cf. *B. amyloruber*). Whether the difference depends upon an unequal rate of disappearance of the enzyme in the two media or whether gelatinase production continues in some cases in the gelatin culture after ceasing in the broth is uncertain.

The failure of the presence of geiatin to provoke the formation of gelatinase does not seem to be peculiar to bacterial cultures. Malfitano,[1] in working with *Aspergillus niger*, found that the kind of enzyme produced by this mold did not depend upon the presence of gelatin or upon the nature of the medium, except in so far as this influenced the general development of the mycelium. And Butkewitsch[2] states that although peptone hinders the action of the gelatinase formed by *Aspergillus* and *Penicillium*, this enzyme is produced abundantly in a medium containing peptone.

Particular interest attaches to the question of gelatinase production in non-proteid media. Fermi[3] averred that most bacteria formed no enzymes upon proteid-free media, only *B. pyocyaneus* and *B. prodigiosus*—among those tested by him—giving positive results. Fermi does not specify the media employed further than to state that his experiments were made "auf Phosphor-Ammonium-Nährsalzen mit Zusatz von Zucker oder Glycerin." The microbes mentioned above were said to produce their enzymes only in the media containing glycerin, not in those with sugar. Katz,[4] on the other hand, was unable to confirm Fermi's observation that *B. megatherium* did not

[1] *Ann. de l'Inst. Past.*, 1890, 14, p. 60.
[2] *Jahrb. wiss. Bot.*, 1903, 38, p. 147.
[3] *Centralbl. f. Bakt.*, 1891, 10, p. 405.
[4] *Jahrb. wiss. Bot.*, 1898, 31, p. 599.

produce any enzyme upon a medium containing glycerin without peptone. More recently Abbott and Gildersleeve[1] have expressed the same conviction as Fermi regarding the production of proteolytic enzymes in non-proteid media. They state that in their experiments the minimum evidence of digestion was given by filtrates from non-proteid culture media. Details of these experiments are not recorded.

Experiments made by me show that under proper conditions a large amount of gelatinase is formed by some bacteria in non-proteid media. The following solutions were employed.

A.	Asparagin	0.2g.
	Na_2HPO_4	0.2
	H_2O (redistilled)	100.0 c.c.

In this very simple medium *B. subtilis* grows well and produces gelatinase slowly, but so abundantly that 0.3 c.c. of the filtrate of a 90 day culture at 20°, 0.1 c.c. of a 120 day culture, and 0.05 c.c. of a 150 day culture brought about complete liquefaction of the standard amount of gelatin (p. 125). The use of the same medium plus magnesium sulphate

B.	Asparagin	0.2g.
	Na_2HPO_4	0.1
	$MgSO_4$	0.1
	H_2O (redistilled)	100.0 c.c.

gave very similar results, but the substitution of a potassium for the sodium salt

C.	Asparagin	0.2g.
	K_2HPO	0.1
	$MgSO_4$	0.1
	H_2O (redistilled)	100.0 c.c.

led to a negative result, as shown by tests made with 0.5 c.c. of the filtrate of a 150 day culture.

D.	Asparagin	0.2 g.	*E.*	Asparagin	0.2 g.
	Na_2HPO_4	0.1		Na_2HPO_4	0.1
	$MgSO_4$	0.1		$MgSO_4$	0.1
	Dextrose	1.0		Lactose	1.0
	H_2O (redistilled)	100.0 c.c.		H_2O (redistilled)	100.0 c.c.

The addition of dextrose had little effect in increasing the gelatinase production in the organisms studied, *B. subtilis* in fact yielding much less in *D* than in *A*.

[1] *Loc. cit*

MINIMUM LYTIC DOSE, *B. subtilis.*

Age of Culture	*A*	*D*
60 days	0.5 c.c. +	0.5 c.c. 0
90 "	0.3 +	0.5 0
120 "	0.1 +	0.3 +
150 "	0.05 +	0.3 +

Lactose, on the other hand, is distinctly favorable to the formation of the enzyme.

MINIMUM LYTIC DOSE.

	Age of Culture	*D*	*E*
B. subtilis	30 days	0.5 c.c. 0	0.10 c.c. +
	60 "	0.5 0	0.05 +
	120 "	0.3 +	0.01 +
B. pyocyaneus	60 "	0.5 0	0.5 +
	120 "	0.5 0	0.3 +

The addition of glycerin (2.0 per cent) to *C* did not give as a rule quite as strongly lytic filtrates as lactose, but the difference was not as great as that between lactose and dextrose.

In the lactose medium, *E*, the M. L. D. was determined as follows:

B. subtilis	30 days	0.1 c.c.
"	120 "	0.01 *
B. fuchsinus	112 "	1.0 †
B. pyocyaneus	120 "	0.3
B. prodigiosus	270 "	0.01
B. ruber indicus	112 "	0.1
B. proteus	112 "	1.0 †
Sp. Metchnikovii	120 "	0.3
"	150 "	0.1
Sp. cholerae	150 "	0.5 †

* This was more strongly lytic than any broth or gelatin culture of this organism observed in the course of any experiment.

† This amount produced only partial liquefaction.

These observations show that in relatively simple synthetic media certain bacteria can produce large amounts of gelatinase, amounts in fact that are quite as great as those produced in broth or gelatin media. This is true, however, as a rule only when growth continues for a long period. The latter fact probably explains the failure of observers to discover the occurrence and extent of enzyme formation in non-proteid media.

Reaction of medium.—The influence of the reaction of the culture medium upon enzyme production is advantageously studied in the case of such an organism as *B. pyocyaneus*. As is well known,

this bacillus is an active alkali-former. The reaction of neutral broth inoculated with *B. pyocyaneus* becomes speedily alkaline, while gelatin inoculated at the same time becomes acid, owing to the acidigenic action of the gelatinolytic enzyme. The two cultures accordingly diverge in their reaction. Enzyme formation, however, occurs in both media and apparently takes place to about the same extent in each. Parallel cultures of *B. pyocyaneus* in broth and gelatin reacted as follows after 24 days at 36°: gelatin 4.0 per cent acid; broth 0.5 per cent alkaline. One-tenth c.c. of filtrate from each culture gave complete liquefaction; 0.01 c.c. was negative for the broth culture, but the same amount of the gelatin filtrate produced a slight softening. In another gelatin culture, 26 days old at 20°, alkali formation had almost completely neutralized the acids arising from the gelatin splitting, and the filtrate in this case reacted neutral to phenolphthalein. The gelatinolytic potency of this filtrate, however, was almost exactly the same as in the instance just cited, viz.:

*0.1 c.c.	+
0.05	±
0.03	−
0.01	0

*In all the tables the following signs are used: + = complete liquefaction; ± = partial liquefaction − = gelatin noticeably softened; 0 = perfectly solid, like control.

In another case a culture of *B. pyocyaneus* in broth was compared with a control culture in gelatin with the following result:

FORTY-EIGHT HOURS AT 37°.

	Broth (+0.2%)	Gelatin (+0.5%)
0.5 c.c.	+	+
0.3	+	+
0.1	0	0

NINE DAYS AT 37°.

	Broth (−0.2%)	Gelatin (+2.5%)
0.05 c.c.	+	+
0.03	+	+
0.01	+	+
0.005	−	−
0.003	0	0

It is evident from these and many other observations of the writer in respect to varying reactions among gelatinolytic filtrates that the production of a gelatin-liquefying enzyme is not very dependent upon

the reaction of the culture medium. It is probable that the reaction is of importance only in so far as it influences the general conditions of growth of the microorganism. The facts to be adduced presently concerning the conditions under which the enzymes manifest their activity tend to support this view.

PHYSICAL CHARACTERS.

Resistance of gelatinases to heat.—The statements of authors are not in harmony on this point. Fermi[1] found that the gelatinolytic enzymes were destroyed at relatively low temperatures: the majority of those tested by him were rendered inactive at 50°–55° C., and all were destroyed at 70°C. Abbott and Gildersleeve,[2] on the other hand, observed a surprising heat resistance on the part of the proteolyitc enzymes elaborated by certain species, and asserted that some were even "capable of exhibiting their characteristic function after exposure in the moist state to a temperature of 100° C. for 15 to 30 minutes." Hata[3] also declares that the enzymes of *B. prodigiosus* and *B. fluorescens liq.* resist high temperatures. None of these authors have given definite data concerning the amount and strength of the enzymes employed in their experiments.

The varying results obtained in some of my earlier work gave rise to the suspicion that differences in the reaction of the medium in which the enzyme was contained were responsible, at least in part, for the divergent statements. It has been pointed out elsewhere in this paper that the reaction of the culture medium is often profoundly affected by bacterial growth, that gelatin cultures of liquefying species are more acid than the corresponding broth cultures, and that the reaction varies according to the particular stage of growth and enzyme action at which it is tested. The influence of the reaction of the medium (presence of H or OH ions) upon the thermal death-point of the enzyme is shown in the experiments which follow. It is unnecessary further to multiply such instances. In almost every case tested it was found that increasing the acidity of the enzyme-containing fluid raised the "heat resistance" of the enzyme, while adding to the alkalinity lowered it. In one case the M. L. D. of an enzyme (0.01 c.c., *B. prodigiosus*) was not affected by boiling for 15 minutes

[1] *Centralbl. f. Bakt.*, 1891, 10, p. 401.
[2] *Jour. Med. Res.*, 1903, 5, p. 42.
[3] *Centralbl. f. Bakt.*, 1904, Ref. 34, p. 208.

in a 5 per cent acid medium, while in a neutral solution 50 M. L. D. were completely destroyed. That acid and alkali are not the only substances whose presence must affect the "heat resistance" of

EXPERIMENT.

BROTH CULTURE *B. prodigiosus.*

UNHEATED.

Amt. of Culture c. c.	2.5% Acid	1% Acid Orig. React.	Neutral	2.5% Alkaline
0.2	+	+	+	+
0.1	+	+	+	+
0.06	+	+	+	+
0.03	+	+	+	+
0.01	+	±	o	o
HEATED 72° FOR 1 HOUR.*				
0.2	+	+	o	o
0.1	+	±	o	o
0.06	+	o	o	o
0.03	±	o	o	o
0.01	o	o	o	o

*In each case 2 c.c. of culture or filtrate was placed in a sealed tube and immersed in a water-bath.

EXPERIMENT.

BROTH CULTURE *B. pyocyaneus.*

UNHEATED.

Amt. of Culture c.c.	3% Acid	1.3% Acid Original	Neutral
0.20	+	+	+
0.08	+	+	+
0.06	+	+	+
0.04	+	+	+
0.02	o	o	o
HEATED TO 70° FOR 30 MINUTES.			
0.20	+	o	o
0.08	+	o	o
0.06	+	o	o
0.04	±	o	o
0.02	−	o	o

an enzyme is obvious. In some cases a neutral broth culture was found to resist heating better than a neutral gelatin culture, that is, 2 M. L. D. of the gelatin culture were destroyed at a lower temperature. Among the great variety of substances produced by bacteria, there must be many whose presence affects the stability of the enzyme when heat is applied. Any accurate determination of the heat resistance of bacterial enzymes is hardly possible when the enzymes are

heated in the cultures in which they are produced, and even the enzymes are separated out as far as possible, there remains sible source of error in the adherent impurities.

Filtration of gelatinase.—Levy[1] has shown that certain e rennet, for example, are retained by the Berkefeld and Cham bougies, while others pass through these filters. A few expe have been made to determine how far gelatinase is removed by filtra tion. A 31 day culture of *B. pyocyaneus*, grown at 37° and reacting 0.9 per cent alk. was filtered at 20°C. with the following result:

	Unfiltered	Berkefeld Bougie 60 m. × 15 m. One filtration
0.05 c.c.	+	+
0.03	±	±
0.02	−	−

Another trial with a different culture of *B. pyocyaneus* was as follows:

	Unfiltered	Berkefeld Bougie 60m. × 50m. One filtration	Chamberland Bougie 200 m. × 20 m. One filtration
0.05 c.c	+	+	+
0.03	+	+	+
0.02	+	+	+
0.01	−	−	−

Several successive passages through a Berkefeld bougie did not remove the gelatinase.

	Unfiltered	BERKEFELD BOUGIE. 69 m. × 15 m.	
		2 Filtrations	4 Filtrations
0.02 c.c.	+	+	+
0.01	±	±	−

A similar result was obtained with *B. subtilis*. (Broth culture, 8 days.)

	Unfiltered	Chamberland Bougie 200 m. × 20 m. One filtration
0.1 c.c	+	+
0.5	±	±
0.3	−	−
0.1	0	0

[1] *Jour. Infect. Dis.*, 1905, 2, p. 1.

In respect to passage through the Berkefeld bougie, therefore, the bacterial gelatinases agree with ptyalin and taka-diastase rather than with rennet (cf. Levy, *op. cit.*).

CONDITIONS OF ENZYME ACTIVITY.

Reaction—It is well known that the acid or alkaline reaction of the medium in which an enzyme is present often exercises great influence upon the activity of the enzyme. As regards the particular enzyme under consideration, it seems to have been generally assumed that the gelatin-liquefying enzymes produced by bacteria were most potent in an alkaline medium. Thus Abbott and Gildersleeve[1] state:

> As is the case for the majority of proteolytic enzymes, be their origin wha it may, we find our filtrates to be uniformly more active when they are of alkaline than of neutral or acid reaction. When acidified they are as a rule inactive. In a simlar manner their production by the growing organism is always more marked in alkaline than in either neutral or acid media, even though the latter is not sufficient to depress growth to any marked extent.

Further details are not given in their paper.

The statement that gelatinase production is more marked in alkaline than in neutral or acid media, evidently needs some qualification in view of the facts set forth in another part of this paper (p. 130). It by no means follows that the initial reaction of the culture medium represents the conditions under which the enzyme is produced. A gelatin culture and a broth culture of a liquefying species diverge in respect to reaction from the moment growth begins to take place, the gelatin culture invariably becoming more acid.

The fact that gelatin liquefied by enzyme action has a strongly acid reaction seems to have been generally overlooked by bacteriologists.* The products of gelatin digestion comprise glycocoll, aspartic acid, and glutaminic acids, and other acid substances. When, therefore, any one of the bacterial gelatinases, or, for that matter, Grübler's pancreatin, is added to gelatin, the substances produced by the enzyme action impart a strongly acid reaction to the liquefied mass. In most liquefied gelatin cultures of bacteria the reaction ranges as high as 2.0 per cent to 3.0 per cent acid to phenolphthalein, and in some cases it is over 4.0 per cent. By the very conditions of its action, then, a

[1] *Loc. cit.*, p. 47.

* I have discussed this elsewhere, *Science*, February 9, 1906, p. 220.

bacterial gelatinase must exercise its effect almost exclusively in an acid medium.

The change in the reaction of the medium, as might be expected, is not confined to the liquefied gelatin, but is communicated by diffusion to the yet unliquefied portions.

EXPERIMENT.

Two hundred c.c. of 10 per cent nutrient gelatin (neutral) were placed in 600 c.c. flasks. These were inoculated on one side and the flasks tipped so that liquefaction took place in only one-half. After incubation at 20° C. for four days the liquefied gelatin was drawn off and the reaction of this and of the unliquefied portion determined.

B. amyloruber.

Liquefied gelatin	1.8% acid
Solid gelatin	0.7 "

B. subtilis.

Liquefied gelatin	2.9% acid
Gelatin removed from directly under liquefied area	0.9 "
Gelatin taken 3 cm. from liquefied region	0.6 "
Gelatin from opposite side of flask	0.4 "

Perhaps one reason why the standardization of nutrient gelatin for plate cultures has not been so successful as could be desired is because after growth begins alterations in reaction occur in varying degrees according to the relative abundance or scarcity of liquefying species.

It follows, too, that the reactions produced by bacteria in ordinary broth are dependent, not only upon the presence of sugar, but to a degree upon the amount of gelatin and similar substances in the medium.

EXPERIMENT.

Neutral gelatin was inoculated with *B. pyocyaneus* and the filtrate was found after four days at 37° C. to be 1.8 per cent acid. The action of the filtrate (18 hrs.) upon carbol gelatin of different degrees of alkalinity was as follows:

Amount of Filtrate	Carbol Gelatin		
	0.7% Acid	Neutral	0.7% Alk.
1.8% Acid—			
0.5 c.c.	+	+	+
0.3	+	+	+
0.1	+	+	−
0.05	+	±	−
0.03	±	−	0
0.01	0	0	0
2% Alk. with N/1 NaOH—			
0.5 c.c.	+	±	±
0.3	+	±	±
0.1	+	±	0
0.05	−	−	0
0.03	−	0	0
0.01	0	0	0

The particular question as to the effect of the initial reaction of the medium upon enzyme production was thus answered in a somewhat unexpected manner.

This experiment was repeated with another lot of carbol gelatin, 0.6 per cent acid, neutral, and 0.5 per cent alkaline respectively, with the same result, namely, the acid and neutral gelatins were more quickly affected by the enzyme than the alkaline gelatin. A comparison of *B. pyocyaneus* gelatinase and Grübler's pancreatin (5 per cent solution in distilled water) resulted as follows:

EXPERIMENT.

Amount of Culture	Carbol Gelatin		
	0.8% Acid	Neutral	0.8% Alkaline
B. pyocyaneus—			
0.3 c.c.	+	+	+
0.1	+	+	0
0.05	−	−	0
0.03	0	0	0
Pancreatin solution—			
0.03 c.c.	+	+	+
0.01	+	+	+
0.005	±	±	±
0.003	0	−	−
0.001	0	0	0

The gelatinases produced by *B. amyloruber* and *Sp. Finkler-Prior* behaved as follows:

EXPERIMENT.

Amount of Filtrate	Carbol Gelatin		
	1% Acid	Neutral	1% Alkaline
B. amyloruber—			
0.3 c.c.	+	+	+
0.1	±	+	+
0.05	±	+	±
0.03	−	±	−
0.01	0	−	0
Sp. Finkler-Prior—			
0.30 c.c.	+	+	+
0.10	±	+	+
0.05	−	+	+
0.03	0	±	+
0.01	0	−	+

Further experiments with gelatin of a wider range of acidity and alkalinity gave the following results:

EXPERIMENT.

Amount of Filtrate	Carbol Gelatin					
	5% Acid	3% Acid	2% Acid	Neutral	2% Alk.	3% Alk.
B. pyocyaneus—						
1.0 c.c.	o	o	o	+	+	+
0.5	o	o	o	+	−	o
0.4	o	o	o	+	o	o
0.3	o	o	o	+	o	o
0.2	o	o	o	+	o	o
0.1	o	o	o	o	o	o
Sp. Finkler-Prior—						
0.3 c.c.	o	o	o	+	+	+
0.2	o	o	o	+	+	+
0.1	o	o	o	+	+	+
0.08	o	o	o	−	+	+
0.05	o	o	o	o	+	+
B. prodigiosus—						
0.3 c.c.	o	+	+	+	+	+
0.1	o	−	+	+	+	+
0.06	o	o	+	+	+	+
0.04	o	o	−	+	+	+
0.02	o	o	o	+	+	±
0.01	o	o	o	±	−	o
0.005	o	o	o	o	o	o

These experiments show that not all gelatin-liquefying enzymes area like in respect to the conditions of their activity; that some liquefy a neutral gelatin quite as rapidly as, or even better than, a slightly alkaline one (*B. amyloruber*, *B. prodigiosus*); that some liquefy even more rapidly in an acid medium than in an alkaline one (*B. pyocyaneus*); and that in one case alkaline carbol gelatin is liquefied more rapidly than a neutral gelatin (*Sp. Finkler-Prior*). Vines[1] has recently shown that the digestion of fibrin by vegetable proteases occurs in some cases with both acid and alkaline reaction and in others is limited to acid reaction. He interprets his results as indicating the existence of two distinct vegetable proteases, one of which belongs to the peptases, although the "vegetable pepsin" differs from animal pepsin in being able in some cases to act in an alkaline medium.

I have already pointed out that the acid reaction developed in the course of gelatin digestion interferes with any strict evaluation of the influence of reaction upon the work of the gelatinases. For example, tubes of carbol gelatin with an initial reaction 2 per cent alkaline were liquefied in 20 hours by the enzymes of *B. prodigiosus* and *Sp. Finkler-Prior* respectively, and both tubes then had a reaction of but 0.4 per cent alkaline. By the action of trypsin for 20 hours

[1] *Annals of Botany*, 1905, 19, pp. 149–87.

at 37° C. carbol gelatin originally 0.05 per cent alkaline was made acid as follows:

5% Trypsin Sol.	Carbol Gelatin
0.003 c.c.	0.1% acid
0.03	1.0 "
0.1	2.3 "

All the bacterial gelatinases tested are able to manifest their activity in a medium more or less acid. It is further evident from the experiments cited above that the initial reaction of the gelatin is not a matter of indifference, that the gelatinases derived from several bacterial species are not alike in this respect, and that it is certainly not true that an initial alkaline reaction presents in all cases the most favorable conditions for the gelatinolytic process.

Temperature.—The temperatures at which the enzymes manifest their maximum activity have been approximately determined in several cases. In every instance the enzymes have produced greater liquefaction at 37.5° than at lower temperatures. This is true even when the microorganism forming the enzyme grows better at a lower temperature. A strain of *Sp. Finkler-Prior*, for example, that grew well at 20° C., but refused to grow at 37° C., gave rise to an enzyme that liquefied more rapidly at 37° than at 20°, more rapidly at 45° than at 37°, more rapidly at 56° than at 45°, and even at 60° liquefied better than at 37°, though not so well as at 56°.

The following results were obtained with a *B. pyocyaneus* culture (M. L. D. at 37° C., 20 hours was 0.01 c.c.):

Amount of Filtrate	One Hour At				
	30°	37°	45°	55°	60°
0.1 c.c.	+	+	+	+	+
0.08	−	−	±	+	+
0.05	o	−	−	±	−
0.03	o	o	−	−	o
0.01	o	o	−	−	o

Another test with a pyocyaneus gelatinase showed that, as in the experiment just cited, slightly more liquefaction was produced at 45° than at 37°; at 65°, however, the activity of the enzyme was distinctly checked but not altogether inhibited.

The gelatinase of *B. prodigiosus* behaved in a similar fashion (M. L. D. 20 hours at 37° was 0.02 c.c.):

Amount of Filtrate	One Hour at		
	37°	45°	56°
0.5 c.c.	+	+	+
0.3	+	+	−
0.2	+	+	−
0.1	−	±	0
0.06	0	−	0
0.04	0	0	0

These experiments show that at least in some cases the bacterial gelatinases exhibit their maximum effect at temperatures considerably above the optimum temperatures for the growth of the organism that produces them. The enzyme activity may even be manifested above the thermal death-point of the bacteria producing the enzyme (cf. pyocyaneus gelatinase at 60°—the thermal death-point of *B. pyocyaneus* is 56°—and *Sp. Finkler-Prior*).

THE ACTION OF FORMALIN UPON LIQUEFIED GELATIN.

Mavrojannis[1] has called attention to what he considers an important fact, namely, that while formalin has a solidifying action upon the gelatin liquefied by certain bacteria, the gelatin liquefied by other bacteria remains permanently fluid, even when subjected to the influence of formalin for long periods. Mavrojannis explains the difference by supposing that different stages occur in the destruction of the gelatin molecule, and that the kind of enzyme that is produced by certain microorganisms stops short with the production of gelatoses (hardened by formalin), while in other cases a different enzyme is produced that continues the digestion to the formation of gelatin-peptones (not hardened by formalin). In the former category are to be put, according to Mavrojannis, *St. pyog. aureus*, *St. pyog. albus*, *B. anthracis*, *B. pyocyaneus*, and *Sp. cholerae*, while in the latter belong *Sp. Denecke*, *Sp. Finkler-Prior*, and *Sp. Metchnikovii*.

I have used the same methods for hardening as those employed by Mavrojannis,[2] namely, introduction of the liquefied cultures (gelatin, 10 per cent) into a tightly closed jar in the bottom of which is a 40 per cent formalin solution; the gas liberated from this solution brings about the hardening in a constant and uniform fashion. I have also added to 2 c.c. of the liquefied gelatin five drops of formalin and then placed the tubes in the formalin jar. The outcome is about the same

[1] *Ztschr. f. Hyg.*, 1903, 45, p. 108 [2] *Op. cit.*, p. 109.

in the two procedures, except that the addition of formalin accelerates the hardening process. The results may be stated briefly:

Eight different species have been tested: *B. pyocyaneus* (nine strains), *B. anthracis*, *B. prodigiosus*, *B. subtilis*, *Sp. cholerae*, *Sp. Finkler-Prior*, *Sp. Metchnikovii*, and *Sp. Denecke*. In each case the results were the same. Young gelatin cultures became solid in the formalin jar, while older cultures of the same species remained fluid even after exposure to the formalin vapor for three months. Cultures grown at 20° always solidified sooner than those of the corresponding age grown at 37°. Different strains of the same microorganism gave different results. For example, the gelatin liquefied by nine strains of *B. pyocyaneus*, all grown at 20° for 21 days, hardened in formalin in 24 hours in one case and in 48 hours in two cases, but was still fluid after three months in the other six. The gelatin liquefied by a three-day growth of *B. prodigiosus* at 20° solidified in three days, but the 15 day growth of the same organism remained fluid at the end of three months.

Mavrojannis[1] has even gone so far as to champion the action of formalin upon liquefied gelatin cultures as a means of distinguishing the cholera vibrio from other species. According to this writer, *Sp. cholerae* manufactures a gelatinase which is capable of digesting gelatin only as far as the stage of gelatoses (solidifying in formalin), while *Sp. Metchnikovii*, *Sp. Denecke*, and *Sp. Finkler-Prior* push the decomposition to the gelatin-peptone stage (permanently liquid). An experiment with the cultures of these organisms in the laboratory collection gave the following results:

EXPERIMENT.

A.

Cultures grown at 20° for 10 days, then transferred to formalin jar; liquefaction approximately the same in all.

Sp. Denecke	Hard in 24 hours
Sp. Metchnikovii	" " 48 "
Sp. cholerae (Wherry, Manila)	" " 14 days
Sp. Finkler-Prior	Not hard in 106 "

B.

Cultures grown at 37.5° for 6 days, then transferred to formalin jar.

Sp. Denecke *Sp. Metchnikovii* *Sp. cholerae* (Wherry, Manila) *Sp. Finkler-Prior*	All liquid after 110 days

[1] *Jour. de physiol. et path. génér.* 1904, 6, p. 273

This experiment gives no support to Mavrojannis' criterion of differentiation.

The only conclusion that can be drawn from all my observations on this matter is that no fundamental distinction between the different bacterial gelatinases exists in the sense alleged by Mavrojannis. Young gelatin cultures and those liquefied by feeble strains solidify when subjected to the action of formalin; the same is true of some cultures grown at 20° as compared with those of the same species grown at 37°. On the other hand, all the old cultures of vigorously liquefying strains, of whatever species, are not hardened by formalin action. In other words, the difference observed is simply one of degree and not of kind.[1]

RELATION BETWEEN BACTERIAL GELATINASES AND BACTERIAL HEMOLYSINS.

The question has been raised recently as to whether the hemolytic action displayed by certain bacterial filtrates is not simply one manifestation of their proteolytic activity. On this assumption the liberation of the hemoglobin is regarded as due to the action of an enzyme upon the stroma of the erythrocytes. Abbott and Gildersleeve,[2] as the result of their studies, reached the conclusion that "one may as reasonably attribute the hemolysis exerted by these filtrates to the action of their proteolytic enzymes upon the stroma of the erythrocytes as to any other factor." These authors take the liquefaction of gelatin as the criterion of proteolytic action, and base their belief on the identity of the hemolytic and gelatin-liquefying properties upon certain general analogies. Opposed to this view are the observations of Buxton[3] and Eijckman,[4] who found that the destruction of blood corpuscles in blood-agar does not proceed *pari passu* with the liquefaction of gelatin. The fact that many bacteria, such as *B. coli*, *B. typhosus*, and others, that are unable to liquefy gelatin can exert a hemolytic action, has been considered an additional reason for not identifying the gelatin-liquefying and hemolytic power of bacterial filtrates.

[1] Since writing the above, a paper by Tiraboschi (*Ann. d' igiene sperim.*, 151, 905, p. 429; Abstr., *Bull. de l'Inst. Past.*, 1905, 3, p. 922) has appeared which supports this position.

[2] *Jour. Med. Res.*, 1903, 10, 42.

[3] *Amer. Med.*, July 25, 1903, p. 137.

[4] *Centralbl. f. Bakt.*, 1901, 20, p. 841.

The following facts constitute further and apparently incontrovertible evidence that the hemolytic and gelatinolytic substances are, at least in a number of bacterial filtrates, entirely distinct. While it is true that the potency of gelatinase in some cases is not entirely destroyed by heating to 100° C. for 10 to 15 minutes (*B. pyocyaneus* and *B. prodigiosus*), it is also true that heating for 30 minutes at 110° C. destroys completely all the power of these filtrates to liquefy gelatin, but leaves absolutely intact their hemolytic power. In the second place, certain filtrates which possess marked ability to liquefy gelatin may be entirely devoid of hemolytic power. A single instance may be given. A seven-months-old culture of *B. prodigiosus* in asparagin-phosphate-sulphate-sucrose solution yielded a filtrate, 0.05 c.c. of which liquefied a tube of gelatin completely in 16 hours at 37.5°. Such a solution has some osmotic action on dog corpuscles, but the addition of 0.4 per cent NaCl renders it isotonic, although not affecting its power to liquefy gelatin. The filtrate is then strongly gelatinolytic, but has no hemolytic effect on dog or rabbit corpuscles, even when 0.5 c.c. is used.

Other examples may be presented in tabular form:

		Filtrate	Gelatinolysis	Hemolysis (Dog Corpuscles)
From 3 months' old broth culture—	*Sp. Finkler-Prior*	0.8 c.c.	None	Complete
	" " "	0.1	"	"
	B. amyloruber.....	0.05	Complete	None
	" "	0.5	"	"
From 7 mos.' old broth culture—*B. pyocyaneus*.		0.5	None	Very strong

It is true, then, (1) that certain hemolytic filtrates, heated at 110° may be robbed completely of their power to liquefy gelatin without evincing any diminution of hemolytic power (*B. pyocyaneus*, *B. prodigiosus*); (2) that a bacterial filtrate may possess gelatinolytic power without being able to produce any hemolysis whatsoever (*B. amyloruber*); (3) that a bacterial filtrate may be strongly hemolytic without possessing any power to liquefy gelatin.

SUMMARY AND CONCLUSIONS.

1. There is no evidence that the presence of gelatin in a culture medium leads to any particularly rapid or abundant production of the specific ferment acting upon the gelatin. On the contrary other

factors are of much greater influence than the presence of gelatin in determining the generation of liquefying enzymes.

2. In simple non-proteid solutions of asparagin, lactose, and mineral salts (sodium phosphate and magnesium sulphate) gelatinase is produced by some bacterial species quite as abundantly, although generally not as rapidly, as in nutrient broth or gelatin. Lactose is more favorable than dextrose to gelatinase production.

3. The reaction of the culture medium is, at least in some cases, without apparent effect upon the enzyme production except as it affects the conditions of bacterial growth.

4. The heat resistance of the gelatinase, as this is determined by heating the ordinary fluid culture, is conditioned by a variety of influences. One of these is the reaction of the medium. The gelatin-liquefying enzymes produced by a number of microorganisms endure heat very much better when heated in an acid than in an alkaline or a neutral medium. The usual tests of heat resistance of bacterial enzymes which are made directly with the culture in which the enzymes are produced have little value.

5. Some, at least, of the bacterial gelatinases pass through the Berkefeld filter without weakening.

6. The reaction most favorable to the manifestation of gelatinolytic activity is different in different cases. The enzymes produced by some species act most rapidly in a medium slightly acid to phenolphthalein, while others do best with an alkaline reaction. It can no longer be maintained that an initial alkaline reaction affords the optimum condition for all bacterial proteolytic enzymes.

7. The enzymes that have been experimented with act more energetically at 45° C. than at lower temperatures. They may continue to be effective at temperatures as high as 60°.

8. Some bacterial enzymes manifest their activity at temperatures considerably above the thermal death-point of the organism producing them.

9. The gelatin liquefied by some cultures of bacteria is hardened by formalin. This, however, is true chiefly in the case of young cultures, of cultures grown at room temperature, and of feeble strains. No difference, such as alleged by Mavrojannis, exists between different species. The stage of liquefaction in which formalin produces

hardening is simply an early stage of digestion, and is followed under favorable conditions in all liquefying species by a state of permanent fluidity.

10. The bacterial hemolysins and bacterial gelatinases are entirely distinct. In no case is there reason to believe that the bacterial gelatinases can produce hemolysis.

A STATISTICAL STUDY OF GENERIC CHARACTERS IN THE COCCACEÆ.*

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ASSISTED BY

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I. PURPOSE OF THE INVESTIGATION.

There has been placed in the hands of the biologist within the last few years a new instrument of research of the highest value. This is the statistical method, first suggested for the study of human characteristics by Quetelet (1846), specifically applied to the biological problems of variation and heredity by Galton (1889), and extended and developed in detail by Pearson and his pupils. The most important papers on this subject may be found in the files of the *Philosophical Transactions of the Royal Society of London* and in *Biometrika*. Admirable brief summaries have been prepared by Pearson (1900) and Bigelow (1904).

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In many fields of science the statistical method, in its strict sense, is not applicable. Where laboratory experiments may be made, as in most fields of physics and chemistry, a comparatively small array of data obtained under perfectly controlled conditions may permit the derivation of laws of relationship without extensive statistical analysis. The same thing is true in certain fields of biological research. As soon, however, as we proceed to the subtler problems of evolution, it becomes necessary to accumulate a large number of observations and to analyze them by recognized statistical methods. These methods alone have brought order out of chaos in anthropology (Ripley, 1899). They have laid the first foundation for a real science of mental and social phenomena (Thorndike, 1904; Woods, 1906). They offer the most promising clue for tracing the true relationships among the lower forms of plant and animal life.

As we have elsewhere pointed out, the classification of the bacteria presents peculiar difficulties.

Morphological distinctions are so slight that physiological characters must necessarily be invoked in order to separate and classify the various organisms, and these physiological characters are often variable. Pathogenicity may be taken as a type of those powers of the organism which are easily and profoundly modified by external conditions. On the other hand, there are numerous characters which appear to be extremely constant. Such minute differences as occur in the resistance of different races to unfavorable conditions often remain unchanged through long periods of cultivation. In using these constant characters for classification we are met by another difficulty. Though constant, the differences are very minute, and in studying a number of organisms a perfect gradation is often found between the widest extremes. This is exactly what should be expected from organisms which reproduce only by asexual methods, since it is the fusion of independent cells which swamps minor differences producing the uniformity of species among higher plants. With asexual reproduction every minute variation which is inheritable must persist unchanged until some other chance variation occurs. Each such variation means a new and different type of bacterium.

The immense number of generations which may succeed each other in a short space of time makes boundary lines as shifting as they would become among the higher plants if a dozen geological epochs were considered all at once.

Since with unicellular organisms acquired characters may probably be inherited in a higher degree than with other forms, existing races of bacteria will be markedly influenced by the selective effect of environmental conditions, and must bear the impress of their recent history.

There are, therefore, no species among the bacteria in quite the sense in which we ordinarily use the word—as indicating a group of individuals bound together by a num-

ber of constant characters and easily identified by mutual fertility. From one point of view each distinct race might be considered a species; but to apply a name for every grade of difference in each varying character would be impracticable; and such names could have no true specific value. The best solution of the difficulty is the establishment of certain types around which the original organisms may be more or less closely grouped; but it must be clearly recognized that the groups thus formed are defined by relation to the type at their center and are not sharply marked off at their extremities from the other groups adjacent.[1]

For these reasons the science of systematic bacteriology has remained in a notably undeveloped state. A score of large groups of bacteria have been more or less satisfactorily recognized by Flügge (1896) and others. Certain of these groups, like the aërobic spore-formers, the colon bacilli, and the diphtheria bacilli, doubtless represent true natural families or genera. In one such group, that of the aërobic spore-formers, where appreciable morphological differences exist, the species and varieties have been carefully worked out by Chester (1904). Far too many specific names among the bacteria however, mean less than nothing. The incomplete description of a vast number of identical or minutely differing forms has led to a confusion quite disheartening to the student of such systematic works as those of Migula (1900) and Chester (1901). Among the Coccaceæ we have compared the published descriptions of 445 species and found evidence for only 31 distinct types (Winslow and Rogers, 1905). These are defined mainly by arbitrary combinations of the three characters of acid production, chromogenesis, and the liquefaction of gelatin. It is small wonder that most bacteriologists have abandoned any attempt at a natural classification, and have sought refuge in such frankly arbitrary schematic groupings as those of Fuller and Johnson (1899), Weston and Kendall (1902), and Jordan (1903). The same tendency carried to its extreme is shown in the decimal systems of Gage and Phelps (1903), and Kendall (1903), and in the modifications recently adopted by the Society of American Bacteriologists.

These systems are most valuable for a routine descriptive work, and for arranging and cataloguing records of cultures. They may, however, lead to error, unless used with due caution. In the first place, the determinations on which such schemes are based are usually qualitative only and not quantitative. In the second place, the

[1] WINSLOW AND ROGERS, 1905.

application to all bacteria of one fixed series of characters arranged in an arbitrary order tends to suggest a mechanical view of bacterial relationships which is very far from the complex truth.

In order to obtain a just idea of the real relations of organisms, it is necessary to consider each systematic group by itself. As Robinson has pointed out in an admirable paper on generic classification (Robinson, 1906), "a difference having great classificatory significance in one place may be almost valueless in another." In studying any one group it is therefore necessary to examine afresh each of the various characters used for the identification of bacteria in general, and to determine its local value and significance. Secondly, under each character it is necessary to determine how many distinct types of structure or function may occur. This can be done only by measuring the character quantitatively in a large series of individuals, and plotting curves of frequency which will show whether the individual forms fluctuate about one or several modes. This has been attempted by Howe (1904) with good results, for the composition of the gas produced in dextrose broth by organisms of the *B. coli* group.

Finally, the correlation between various properties should be determined, since it is obvious that the presence of several distinct characters in association is generally of more significance in classification than that of any one alone.

In the present study we have attempted to obtain the data indicated, for certain groups of the Coccaceæ. We have measured the easily and definitely measurable, variable characters in 500 separately isolated races of organisms, and analyzed the data obtained, with two ends in view. We have first plotted the frequency curve for each character to find whether the array varies about one or several modes, and where the modes are situated, with some measure of the extent of variation about these centers. In the second place, we have calculated correlation factors for the most significant pairs of characters. Each mode on the curves of frequency may fairly be taken to mark a natural species or variety, and the characters which vary together must form the most important basis for the establishment of the larger groups. By such a method alone it is possible to locate those mountain peaks in the chain of bacterial

variations which rightly deserve generic and specific names, although records of the characters of individual races by the decimal system are of the greatest value in mapping out intermediate regions. Only the statistical study of numerous individuals by comparable quantitative methods can reveal the general laws of natural classification among the bacteria; and this study must be made in each group with an open mind free from arbitrary predispositions.

We desire in advance to deprecate a comparison between the present work and the numerous detailed and exact biometrical studies which have appeared in other fields. In bacteriology our methods of measurement are crude and tedious, and the general knowledge requisite for the selection of a homogeneous mass of material is lacking. We should know the outlines of the general groups of the cocci, for example, before we can properly select material to study variation in any one of them.

II. METHODS OF THE INVESTIGATION.

1. ISOLATION OF CULTURES.

With regard to the larger groups of the Coccaceæ we have elsewhere shown (Winslow and Rogers, 1905) that the family could be divided into two subfamilies and five genera, defined as follows:

Subfamily 1, Paracoccaceæ (Winslow and Rogers): Parasites (thriving only, or best, on, or in, the animal body). Thrive well under anaërobic conditions. Many forms fail to grow on artificial media; none produce abundant surface growths. Planes of fission generally parallel, producing pairs, or short or long chains.

Genus 1, *Diplococcus* (Weichselbaum): Strict parasites. Not growing, or growing very poorly, on artificial media. Cells normally in pairs surrounded by a capsule.

Genus 2, *Streptococcus* (Billroth): Parasites (see above). Cells normally in short or long chains (under unfavorable cultural conditions, sometimes in pairs and small groups, never in large groups or packets). On agar streak effused, translucent growth, often with isolated colonies. In stab culture little surface growth. Sugars fermented with formation of acid.

Subfamily 2, Metacoccaceæ (Winslow and Rogers): Facultative parasites or saprophytes. Thrive best under aërobic conditions.

Grow well on artificial media, producing abundant surface growths. Planes of fission often at right angles; cells aggregated in groups, packets, or zoöglea masses.

Genus 3, *Micrococcus* (Hallier) Cohn: Facultative parasites or saprophytes. Cells in plates or irregular masses (never in long chains or packets). Acid production variable.

Genus 4, *Sarcina* (Goodsir): Saprophytes or facultative parasites. Division under favorable conditions in three places, producing regular packets. Sugars as a rule not fermented.

Genus 5, *Ascococcus* (Cohn): Generally saprophytic and cells imbedded in large, irregularly lobed masses of zoöglea, in process of carbohydrates. Acid usually formed.

In the present investigation we have included representatives of only three of these genera. The organisms belonging to the genus *Diplococcus* do not lend themselves to comparative study on account of the difficulty with which they may be cultivated, and representatives of the genus *Ascococcus* occur, if at all, only in certain peculiar habitats. We have limited our study to forms which can be found in ordinary environments, and which may be cultivated on ordinary laboratory media; that is, to the genera *Streptococcus*, *Micrococcus*, and *Sarcina*.

We have procured our cultures in approximately equal proportions from five different sources: from the internal tissues of the diseased human body, from the outer surfaces of the normal human body, from water, from earth, and from air. Cultures classed under Habitat I, the tissues of the diseased body, were obtained chiefly from the Boston City Hospital, and the Massachusetts General Hospital, of Boston, and the Johns Hopkins Hospital, of Baltimore. We desire to express our cordial thanks to the bacteriologists of these institutions for their courtesy in furnishing us with these organisms. The cultures classed under Habitat II, surfaces of the normal body, were obtained from three sources. A considerable number were isolated from serum tubes, received by the Boston Board of Health for diphtheria diagnosis. In this connection we desire to acknowledge the courtesy of the bacteriologists of the Board. Only those cultures which gave a negative diagnosis for diphtheria were used. Another series of cocci was isolated from the hands of students

in the Massachusetts Institute of Technology. In collecting them each subject rubbed the front and back of one hand with a wet wad of sterile cotton, running the wash water into a sterile cup. Finally a small number of cultures were obtained from excreta of man and animals. Under Habitat III cultures were obtained from a wide variety of natural waters—public supplies, streams, ponds, pools, shallow wells, driven wells, and the sea. Samples were taken as far as possible only from sources held to be free from pollution. Under Habitat IV organisms were isolated from various samples of earth, loam, clay, sand, etc., obtained mainly in different regions of eastern Massachusetts. The cultures grouped under Habitat V were taken from plates exposed to the air, indoor and out, and here are also included certain organisms of unknown origin which appeared as contaminations, or for whose previous history we have no record.

In each case the sample to be studied was first plated on agar and incubated at 20°. Colonies which looked like cocci (not possessing, that is, the characters of such well-marked forms as *B. mesentericus*, *B. Zopfi*, or *B. fluorescens*) were fished to agar streaks; from each sample only one culture was taken, unless several distinct types of colonies appeared. The agar streak cultures were examined under the microscope and, if apparently cocci, were replated in order to insure their purity, again transferred to agar streaks, and again examined under the microscope. All this preliminary work was carried out at 20°, and the stock cultures finally obtained were kept on agar at the same temperature. There can be no doubt that by this method of procedure we failed to obtain many of the more strictly parasitic streptococci which grew only feebly on solid media and are most active at a temperature of 37°. This fact must be taken into account in interpreting our results. For *Micrococcus* and *Sarcina*, however, the series should be fairly representative.

2. Selection of Characters for Study.

The characters ordinarily used in descriptive bacteriology are few, particularly in a group of such simple morphology and limited biochemical powers as the Coccaceæ. This number must be still further reduced, however, when we come to inquire which of them really indicate constant and independent variations. In the first place, it

is necessary to eliminate properites which are due mainly to the character of the medium and the conditions of incubation. As we shall show later, those minute differences in the appearance of colonies on gelatin which form the basis for a large number of German descriptions, fall mainly under this head. Secondly, many characters, while really belonging to the organism itself at a given moment, are so easily modified by cultivation under other conditions as to be practically worthless in systematic work. Among the cocci, pathogenicity is a property of this sort. In the third place, it is evidently unfair to give independent weight to characters which are simply the indirect result of other properties already recorded. Thus among the cocci differences in broth cultures are closely connected with the size of the cell aggregates. Organisms growing in large groups, like most of the sarcinæ, produce heavy sediment and often colony-like groups on the walls of the tube, while those in which the cells readily separate exhibit a more diffuse turbidity. Plate cultures add little more information than may be obtained by a careful scrutiny of stabs and streaks; and the growth on potato and blood serum in many groups of bacteria, and particularly among the cocci, are only valuable as measures of that extremely fugitive quality, the general vigor of the culture.

The considerations which have influenced us in the selection of characters for study among the Coccaceæ may be conveniently arranged in the order, and under the headings, of the Report of the Committee on Standard Methods of Water Analysis to the Laboratory Section of the American Public Health Association (1905).

3. MORPHOLOGICAL CHARACTERS.

Form.—The form of the individual cell furnishes no help in the classification of the Coccaceæ, since under favorable conditions all appear as regular spheres. Irregular oval forms occur at times, particularly in cultures freshly isolated from the throat or alimentary tract, but the form usually becomes normal after cultivation.

Manner of grouping.—The grouping of the cell elements offers a character of considerable importance among these bacteria. While the cocci do not exhibit an entirely unchanging form of grouping, the individuals do show a distinct tendency to occur in one of four forms—either in pairs, chains, masses, or packets.

The grouping is somewhat influenced by the age of the culture and by the kind of medium on which it has grown. Even the same culture will show wide variation from the typical arrangement of the elements. For instance, streptococci occur singly, in pairs, chains, and small masses; but the most frequent arrangement, and that obtained under the most favorable conditions (in liquid media), is in chains. Again, sarcinæ occur singly, in pairs, and in small masses as well as in packets, yet the typical form is the sarcina-packet. Cocci grown on Nährstoff regularly occur in plates, and usually capsulated ones.

In a number of preliminary studies we compared the groupings of the same cultures in various media and under various conditions, examining cultures of different ages, from nutrient broth, sugar broth, peptone solution, hay infusions, nutrient agar, and gelatin, and acid and alkaline gelatin. Cultures more than two weeks old showed abnormalities both in the individual cell and in its groupings. With this exception, the differences produced were very slight. The only constant effect of the medium upon grouping which was apparent was a more distinct development of chains in liquid cultures. Organisms which appear as long chains in fresh broth cultures may show only short chains with irregular groups on solid media. In the present study we have omitted the broth morphology for lack of time, and have recorded the grouping only as apparent on the agar streak.

The streaks used were never more than three days old, and the grouping was observed after staining lightly with methylene blue and mounting in cedar oil. Too heavy staining may introduce a serious error by making packets of small sarcinæ appear like large single cells. These observations on the culture stained with methylene blue were controlled by careful observations of the slides prepared for the study of the Gram stain, as noted later.

We have distinguished two main groupings only by this method of examination. The occurrence of packets marks one, and the absence of packets the other, group. In the first group occur the streptococci, which produce pairs, long chains, and irregular groups; and the micrococci, which show pairs, short chains, fours, and irregular groups; while the sarcinæ include organisms which produce fours, irregular groups, and packets, as well as those

extreme forms which show only packets. None of these differences but that between the presence and absence of packets appear on agar with sufficient constancy to be determined definitely. For distinction between streptococci and micrococci the observation of broth cultures would perhaps be valuable.

Dimensions.—The cocci exhibit a range in size from 0.1 to 2.0 μ with considerable variation between individual cells in the same culture. We were somewhat surprised to find that we could demonstrate no definite relation between size and the age of cultures, or the conditions of cultivation. In a series of preliminary studies the same organism was grown on seven kinds of media and examined at intervals during a period of two months. The maxi-

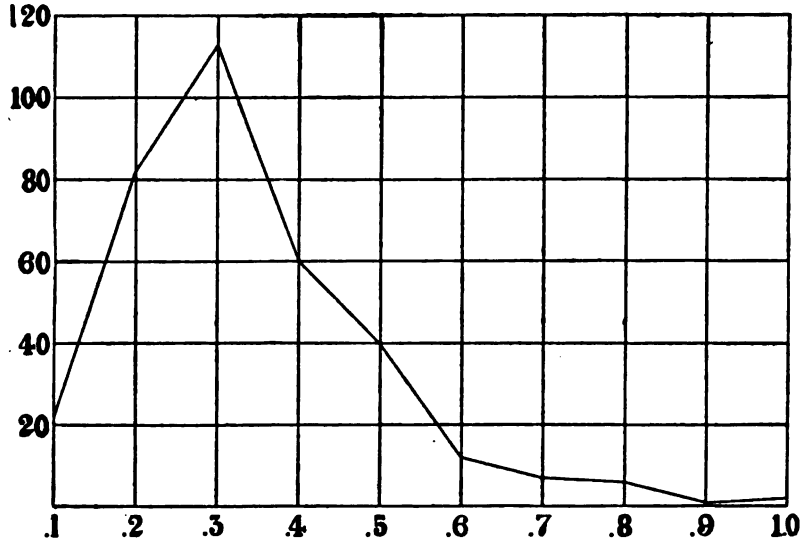

FIG. 1.—Dimensions of 345 cocci. Abscissæ, average diameter in μ. Ordinates, number of cultures.

mum size, in different cultures, was recorded on the first, second, seventh, 14th, 42d days, and after two months respectively. The maximum size developing in the different kinds of media during those two months was found, respectively, in broth at 37°, broth at 20°, Nährstoff-Heyden, nutrient gelatin, acid and alkaline gelatin, and under anaërobic conditions. In other words, the age and kind of medium had no constant effect, except that in most cases the Nährstoff and other poor media showed the smallest individuals. No

constant difference in size was apparent in comparing solid and liquid cultures. One series of organisms examined in dextrose broth and on agar, at periods ranging from one day to two weeks, showed the same average size in both media and at all ages. Finally we attempted to see whether prolonged cultivation under special conditions would affect the size of the cell. Cultures were grown for 10 days, in broth at 37°, on nutrient gelatin, and on acid and anaërobic gelatin with daily transfers. The size of each culture was recorded on the 10th day, after which time each was transferred to gelatin and examined after one day. The results showed practically no significant differences.

In a comparison of the size as determined by examination of living organisms and of stained preparations, the cells appeared generally somewhat smaller after staining. This is no doubt partly due to some shrinkage in drying, and partly to the imperfect definition which makes the unstained specimens appear larger than they really are. Occasionally, when the staining was too heavy, the stained cells appeared larger. In any case the differences are unimportant, and we have used the size of the methylene-blue-stained preparation throughout our work.

Staining reactions. — Since the cocci, as far as we have examined them, all stain easily with methylene blue, we have made no special tests with anilin-gentian-violet. The Gram stain has, however, been used on all our cultures, since, in the genus *Diplococcus* and in many other groups, it has been thought to have such special importance.

The value of this staining method has been studied with considerable care by Mr. A. T. Brant, working in the laboratories of the Institute. Mr. Brant found, as other observers have done, that while certain bacteria are constantly Gram-negative or Gram-positive, others exhibit an intermediate condition, retaining the stain under some conditions and giving it up under others. In his, as yet unpublished, paper he notes, for example, that all cultures of *B. coli* are decolorized by one minute's treatment with alcohol, while *B. megatherium* constantly fails to decolorize after three hours. On the other hand, with *B. fluorescens*, *M. pyogenes*, *M. aureus*, and *B. diphtheriae* the result is affected by the time of decolorization, as well as by the age of the cultures. Between the fixed points at the

extreme, preparations will yield varying results, showing some cells stained and others decolorized. As a rule, the large majority of cells in a given preparation will show one reaction or the other; but a second slide made from a similar doubtful case might yield a different result.

The time chosen for decolorization is, of course, an arbitrary factor which will affect the proportion of positive results obtained. In our work, as a result of Mr. Brant's experiments, we fixed on three minutes, although we are not certain that this is really preferable to the five-minute period fixed by the Committee on Standard Methods. We have applied the anilin-oil-gentian-violet for one and a half minutes, and the Gram solution for one and a half minutes instead of the one- and two-minute periods of the committee.

In all cases we made the stain on young 20° agar cultures (not over five days old), and in each case the test was made in duplicate at different times. When the results of the two tests coincided, the culture was recorded as positive or negative. Cultures which gave one positive and one negative test, or in which the stained and decolorized appeared in about equal proportions, are recorded in an intermediate class.

Flagella.—As a result of the work of Ellis (1902), we have devoted considerable time to the study of motility among the cocci. This author reported the finding of spores and flagella in various streptococci and sarcinæ, and Arthur Meyer carried this position to an extreme in the statement that "the researches of Ellis have rendered it doubtful whether there are any species of bacteria which entirely lack flagella" (Meyer, 1903). We examined a number of cultures very carefully, transferring them at frequent intervals on different media, according to the general plan adopted by Ellis. We found in almost every case active vibratory movements, with a tendency to incomplete rotation, the successive jerks sometimes producing a gradual translation across the field. This type of behavior is entirely different from the true motility characterized by slow, steady revolution, which appears in such forms as *S. agilis*. We are convinced that most of the cocci are non-motile, while a few forms show true movement; it is with this type of motility that clearly stainable flagella have been found associated. The study of this character is

therefore of significance. It is questionable, however, whether it is one of the most important characters in this group of bacteria. It appears from the published descriptions of species that this property is not correlated with any other character, arising independently in forms exactly resembling non-motile forms in every other respect. On account of its rarity and this apparent lack of correlation with other differences, as well as on account of the difficulty of studying it, the property of motility has been so far omitted from the present study.

Spores.—The experiments carried out by Ellis (1902) strongly suggest the presence of specially resistant cells in old cultures of the cocci. His figures are, however, by no means conclusive as to the existence of true spores. In the absence of any observations as to germination, we have not felt that the evidence warranted extensive microscopic study of this character.

Fission.—A study of the conditions influencing the growth-forms of the Coccaceæ should be of considerable interest. Pairs and chains are apparently associated with meager, and groups and packets with more abundant, development. The effect of the general rate of growth must, however, be modified by the rate at which cell-wall and cell-protoplasm, respectively, are formed.

A careful study of the method by which these groupings arise in cell-division, such as could be made by the use of Hill's hanging-block method, would no doubt throw much light on all such points, and should precede any final conclusions as to the relationships of the cocci. In examining a large number of organisms, however, the agar block would have proved too time-consuming. We have therefore limited ourselves to the observations made on stained preparations from ordinary cultures.

Capsules.—Considerable preliminary work failed to indicate any constant differences in capsule formation among the cocci studied. This character appears to be of considerable value among the diplococci (Buerger, 1904); but even with them it varies markedly with the medium used for cultivation. We cultivated certain selected organisms in broth at 20° and at 37°, on nutrient gelatin, acid gelatin, alkaline gelatin, anaërobic gelatin, and Nährstoff-Heyden agar, and examined them at intervals by Welch's staining method. In every case capsules were apparent at some stages, being most

strongly developed in old cultures and on poor media like the Nährstoff agar. This character has not seemed to us of sufficient diagnostic value to be included in our routine examinations.

Involution and degeneration forms.—In numerous examinations of old cultures we found no involution forms of special significance. As noted above, swollen and oval forms are more apt to occur in old cultures of cocci, but they are not sufficiently definite to warrant record.

4. CULTURAL CHARACTERS.

In a study of this sort we have necessarily included only those tests which reveal definite and independent variable characters. Most of the commonly observed cultural characteristics are the secondary results of a few fundamental properties which can be observed on one medium as well as on several. For this reason we have eliminated a number of the ordinary media from our routine. The general character of the growth is approximately the same on *agar, blood serum, potato or Nährstoff*, except that agar has always markedly more growth and potato often none. An organism producing abundant chromogenic growth on agar will give good growth and some pigment on the other media. The streptococcus growth on agar gives restricted and veil-like growth on serum and Nährstoff, and usually no growth on potato. In other words, Nährstoff agar, serum, and potato are simply poorer media than agar, and show no specific characteristics other than those due to feebleness of growth. Blood serum may be useful in other groups to show a special type of liquefaction, but in a preliminary study of 50 of our cultures we never found this to occur, and it is nowhere recorded in published descriptions of the Coccaceæ. In 25 out of 50 cultures grown on potato no growth occurred, and in no case have we observed discoloration. These media have therefore been omitted. This action is in accordance with the conclusions of the Committee on Standard Methods (1905), in considering their value for general diagnostic use.

Nutrient broth.—In the group of the cocci we have not found that any information of definite value could be derived from a study of broth cultures. None of the forms studied form a surface pellicle or produce any characteristic odor. There remain to be observed only two features—turbidity and sediment—which in our

judgment depend directly on other properties, such as the general vigor of growth and the size of the cell aggregates. Both turbidity and sediment vary markedly with the age of the culture; what is first turbidity later settles to form sediment, as the waste products of the bacteria check their development. The amount of either depends on the activity of growth. A constant difference often appears between cultures which early in the course of development show considerable turbidity with little or no sediment, and those which almost at once develop a heavy sediment with colony-like masses

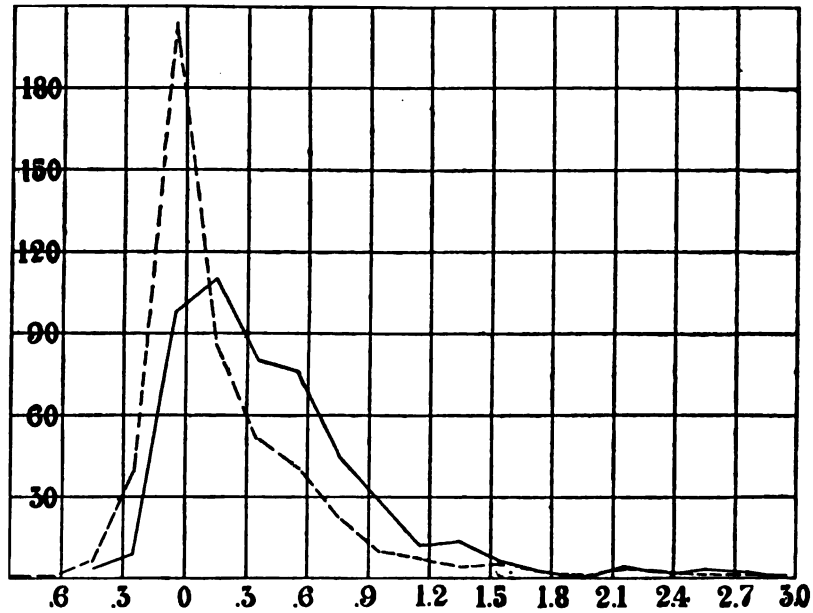

FIG. 2.—Acid production of 500 cocci; in dextrose broth =————, and lactose broth = - - - - - -, Abscissæ, acidity in per cent normal. Ordinates, number of cultures.

of growth clinging to the walls of the tube. This difference, however, appears to be correlated with the growth-form and general vigor of the coccus. Organisms of the Streptococcus type with cells separating readily, which show faint surface growth, produce chiefly turbidity; while organisms like Sarcina with large cell aggregates and rich surface growths, show heavy sediment.

Gelatin plates.—Minute differences in the macroscopic and microscopic appearance of colonies on gelatin are given great

weight in German systems of classification. Certain special characteristics do, indeed, appear in old gelatin colonies of the cocci after several weeks of incubation. Colonies may remain almost spherical; or they may expand in flat, disclike growths with terraced edges. Sometimes a distinct boss appears at the center, surrounded by a flatter area. The edges may be entire, or more or less deeply scalloped, and the edges of the scallops may be produced inward in folds. Concentric rings sometimes appear in the interior of the colony, or zones of partially liquefied gelatin around its periphery. Some of these characters vary without any apparent reason, as different colonies on a plate show different characteristics; this is perhaps due to differences in the position of the original cell relative to the gelatin surface. Most of them are profoundly modified by variations in the amount of moisture in the gelatin and in the atmosphere above. In a series of comparative studies with different conditions of incubation we found that highly characteristic colonies of granular structure, with deeply lobed edges and indented surfaces, could be produced by cultivation in an incubator whose atmosphere was kept dry by calcium chloride. Dunham (1903) has pointed out the wide differences which may be due to slight variations in the physical properties of the gelatin used. Those differences which are really characteristic of the organisms themselves appear to be related to two fundamental powers: the general vigor of growth and the liquefying power. It may be possible that other differences exist in old gelatin colonies which are really characteristic, but in the present state of knowledge it seemed best to omit the gelatin plate in favor of more definite tests. Liquefying power and general vigor of growth are observed in the gelatin stab and the agar streak respectively.

Gelatin tubes.—All our cultures have been studied in the gelatin tube, but only the single character of the amount of liquefaction has been systematically recorded. The distinction between different non-liquefying colonies lies in the amount of surface growth and the color, both of which characters are more easily studied on the agar streak. The character of the surface growth, like that of the gelatin plate colony, does not appear in this group to offer any character of diagnostic value, and all the cocci grow fairly well in the stab.

Among the liquefying forms we have not found the shape of the liquefaction of sufficient constancy to be recorded. Whipple (1902) has strikingly shown the uncertainty of this character—almost every possible type appearing in media made with slightly different commercial gelatins. The Committee on Standard Methods (1905) has also omitted this property.

The amount of liquefaction of gelatin was therefore the only character recorded on the gelatin stab. The method by which this was measured will be described under "Biochemical Reactions."

Agar plates.—The same reasons which led us to omit the gelatin plate militate against the use of the agar plate as a diagnostic test. Constant differences which exist between colonies are slight and depend on a few fundamental properties which may be more easily observed on other media, notably on the agar streak.

Agar tubes.—The general conclusion from what has been said in this discussion of cultural characteristics is that in the cocci a single medium is sufficient for their determination. We should, however, deprecate any extension of this conclusion to other groups where the gelatin stab or the plate culture may yield information of definite value. Even among the cocci further study may show constant and characteristic differences in gelatin colonies, and if this should be the case, no one could fail to welcome an addition to the meager list of diagnostic characters at our disposal. In the absence of evidence as to the value of these media, we feel it unwise to repeat tests mechanically and without any definite purpose, merely because they have had an important place in the historical development of the science.

All cultural characteristics have therefore been observed in the agar tube. A combined streak and stab was made on a slant surface, and the cultures were uniformly studied after incubation for two weeks at 20° C. Cultures of different age exhibit marked differences, but the characters of the old cultures are the outcome of those of the new. Comparative studies with lactose agar and glycerin agar showed neither to be as favorable a medium as ordinary nutrient agar.

In order to obtain a comparative idea of cultural characters we examined two weeks' agar streaks of the whole 500 cultures

at the same time. We are somewhat surprised to find that the visible differences between the cultures were due almost wholly to two properties—chromogenesis and the general vigor of surface growth. There was a distinction in luster between a large majority of the

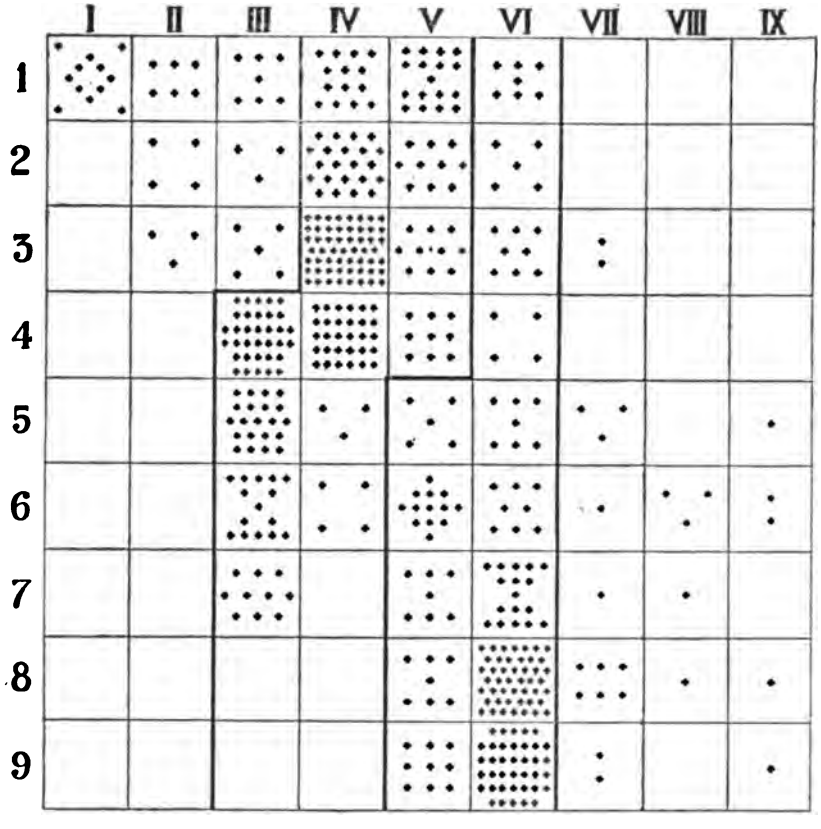

FIG. 3.—Distribution of 500 cocci according to chromogenesis. Roman numerals, hues; Arabic numerals, chromos.

cultures which had smooth and shining surfaces, and a few which were dull and rough. This difference appears, however, to be due simply to the relative amount of growth and moisture in the tube. Faint growths are moist and shining, while heavy growths in tubes which do not contain much moisture show the dry, rough, dull appearance. The "white chromogens" showed another slight difference, varying from an opaque porcelain white to a duller and

more translucent growth of indefinite color and somewhat shiny appearance; but there was no sharp boundary to separate the types. For the present we have omitted this character, although it may prove to be of importance in more detailed work.

We have therefore noted, as cultural characters on the agar streak, only the color production and the vigor of surface growth. The method of studying the former character will be described under "Chromogenesis." Under "Vigor of Surface Growth" we found it possible to distinguish five different types. Grade 1 includes forms of the Streptococcus type which form only a very faint, veil-like, growth, or a few translucent dotted colonies on the surface. Grade 2 is reserved for a somewhat more abundant, but still meager, growth. Grade 3 corresponds to a good, but not abundant, streak; Grade 4, to an abundant growth; and Grade 5, to a very heavy surface development.

The relation to free oxygen is distinctly involved in the vigor of surface growth, and the agar streak also served for the study of various other biochemical reactions. Inhibition of growth by acidity and alkalinity of media, temperature relations, and pigment formation were all recorded on this medium under conditions to be described below.

5. Biochemical Reactions.

Action upon milk.—Milk is a favorable nutrient medium for bacterial growth because of its rich food properties, and in many groups it gives important information, but it has no specific diagnostic value for the Coccaceæ, as all the changes it undergoes are correlated with those which occur in sugar broths and with the general activity of the organism. No coagulating enzymes and casein-digesting enzymes are found in this group, so far as we are aware, and no gas or odor is produced. The only changes which the cocci effect in milk are therefore the production of acid or alkali, coagulation and decolorization of the litmus.

Decolorization has no significance, except that it indicates the general activity of the organism. When the organism is most active, it uses up the oxygen and reduces the litmus, which is accordingly decolorized, and, conversely, when activity grows less, oxygen diffuses from the surface making the litmus pink again.

Coagulation depends upon the amount of acid produced, and is more easily studied in sugar-broth cultures.

Action upon carbohydrates.—The characteristics usually observed in sugar broth are turbidity and sediment, relation to oxygen, gas production, and acid production. We have given reasons, in discussing nutrient broth, for considering turbidity and sediment unimportant, and the relation to oxygen is most sharply defined by surface growth in the agar tube. None of the cocci, so far as known, produce gas, and there remains only acid production to be recorded. For this purpose ordinary straight tubes were used. The sugars tested were dextrose and lactose. Saccharose has been omitted for the present, for lack of time. A preliminary test indicated that this sugar is less commonly fermented by the cocci than are dextrose and lactose.

The media were made up in the usual manner with 2 per cent of the sugar to be tested. The reaction was made about neutral, and after tubing and sterilization it was usually between 0.5 and 1.0 per cent. After standing for two weeks sterile blanks showed a slight further rise in acidity, so control tubes were always kept with each batch inoculated and titrated at the end of the experiment. After considerable preliminary experimentation, it was decided to titrate with phenolphthalein as an indicator in the cold. Methyl orange is not sensitive to the organic acids and gives a poor end-point. With phenolphthalein a comparative series of titrations made on the same tubes, first cold and then boiling, showed slightly higher results by the latter method. Evidently heating increases the apparent acidity more by the breaking-up of unstable compounds than it decreases it by driving off carbon dioxide. The cold method was therefore used. To 5 c.c. of the sugar broth, grown for two weeks at 20°, was added 95° c.c. of distilled water and two or three drops of phenolphthalein. This was titrated against $\frac{N}{20}$ NaOH and from the value obtained was subtracted the acidity of the blank controls titrated at the same time. All tests were made in duplicate, and the final value recorded as the acid or alkali production of the organism is the difference between the average of two titrations of tubes in which it had grown for two weeks and

the average of two blank controls. No determination was made of the rate of acid production as distinguished from this total final acidity, though such observations might be of much interest.

Action upon nitrates.—Data with regard to the reduction of nitrates by the cocci are extremely meager, the presence or absence of this character being recorded in very few of the published descriptions. It seems, however, to have a fair degree of definiteness, and we have included it as a qualitative test in our routine. Each organism was inoculated into a series of 10 tubes of standard nitrate solution. After seven days' growth at 20° the tubes were tested for nitrites and ammonia by the regular method prescribed by the Committee on Standard Methods (1905). The test for nitrates was omitted after it was found that all the cultures, out of a considerable series tested, gave positive results, without exception. The results of the tests for nitrites and ammonia are expressed in the number of tubes which gave positive results, out of the 10 which were tested. In view of the fair constancy of the reaction as observed, we regret that this test was not made quantitative.

Production of indol.—A preliminary examination of some 50 cultures showed no production of indol in any case, and a study of the literature of the cocci indicates that this property is very rare, if it ever occurs, in this group. It was therefore omitted from our routine.

Inhibition of growth by acidity and alkalinity of media.—This character is of considerable importance and warrants careful study, but it is obviously a difficult property to observe in a large series of cultures, and we have not attempted to use it in the present investigation. A preliminary examination of 33 cultures, the results of which are shown in the table, indicated that 1 per cent is the optimum acidity for a majority of these organisms, and that an excess of acidity over this amount is more generally fatal than an alkaline medium.

OPTIMUM REACTION FOR GROWTH AND COLOR PRODUCTION.

NUMBER OF ORGANISMS.

Optimum Reaction	−1.0	−.5	0	+.5	+1.0	+1.5	+2.0
Growth	2	4	5	6	9	6	1
Color	3	1	0	3	9	6	3

Relation to free oxygen.—The Committee on Standard Methods (1905) recommends that the relation of bacteria to oxygen be studied by the comparison of cultures made under normal, and under anaërobic, conditions. A preliminary study of 50 cultures made in this way led to the belief that such a procedure is unnecessary among the cocci. All but two of the cultures studied showed some growth under anaërobic conditions, but the growth was in most cases meager. It became evident that there are two main types of organisms: those which, like Streptococcus, grow only feebly on the surface of aërobic agar, and which grow equally well under anaërobic conditions; and those, like Sarcina, which form abundant surface growths under aërobic conditions, and under anaërobic conditions grow feebly like Streptococci. In other words, there is little difference between the anaërobic cultures of the cocci. Therefore, for purposes of classification we have considered the study of the aërobic surface growth a sufficient measure of the relation to free oxygen, as well as of general vigor. The five grades recorded under vigor of surface growth correspond fairly well to four grades of aërobiosis, from forms anaërobic and facultatively aërobic, to forms which are strong aërobes.

Temperature relations.—There are two points of special importance which ought to be determined in studying temperature relations, the optimum temperature and the high death-point. The death-point at extremely low temperatures is too indefinite to be attempted, and the extreme limits of growth, although desirable data may be omitted as less important than the other two properties.

For the determination of the optimum temperature we first made a series of preliminary studies by comparing agar cultures grown at 10°, 20°, 37°, 45°, and 56°. We found two cultures growing better at 20°, 18 developed equally well at 20° and 37°, 22 showed an optimum at 37°, two grew equally well at 37° and 45°, and four grew best at 45°. These conclusions refer only to the amount of growth, color production being in most cases most active at 20°. From these results we concluded that the information to be gained by cultures grown below 20° and above 37° would be scarcely commensurate with the labor involved, and we have limited our observation to the comparison of growth and color production at 20° and 37°.

Cultures were grown for this purpose on agar at 20° for two weeks and compared by inspection. Amount of growth and depth of color were recorded in five arbitrary grades as follows: growth or color production, much better at 20°, somewhat better at 20°, equal at the two temperatures, somewhat better at 37°, and much better at 37°.

Thermal death-points were included in the original plan of our experiments and have now been made on 87 cultures. The process used is to inoculate from three- to five-day-old agar cultures into broth tubes brought to the desired temperature in a water-bath heated electrically by a platinum coil, and to expose them for 10 minutes. The tubes are then cooled and incubated at 37° for six days. At the end of that time, streaks are inoculated from the broth tubes in order to make sure by characteristic growth that the organisms orginally inoculated are present. Tests are made from 55° up to the point where growth fails. The process is so tedious that we have been unable to complete the work, and must omit this property for the present. The general results so far obtained are as follows:

THERMAL DEATH-POINTS.

NUMBER OF CULTURES KILLED AT VARIOUS TEMPERATURES.

Temperature	50°	55°	60°	65°	70°	75°	80°
Cultures	2	5	24	17	16	22	1

Pigment formation.—The production of color by the bacteria is not only markedly affected by contemporaneous conditions of cultivation, but may be profoundly modified by selective action or by the effect of antecedent environment. First, of the conditions which temporarily affect the production of color, without modifying the inherent chromogenic power of the organisms, may be mentioned the medium, the presence of free oxygen, and the temperature. In some bacteria, media of low nutritive value, like potato and Nährstoff, appear to favor pigment formation, but with the cocci this is not generally the case. Agar has, on the whole, shown a better development of chromogenesis than any other medium tested. The presence of free oxygen is generally an essential for color production, stab growths being almost invariably lightly colored. We have found a single exception to this rule in a coccus which produces

an orange pigment of much deeper tint in the stab than on the surface. In comparing color at different temperatures we have found, in general, a much better pigment formation at 20° than at 37°. A deep orange growth at the lower temperature may often correspond to a white one at 20°. This effect has been recorded in our routine studies, and will be more fully discussed later with their results as a basis. Besides these temporary modifications of the chromogenic power, the actual color of cultures may be indirectly affected by certain other factors. The general vigor of growth is naturally correlated with apparent depth of color, and the dryness of the atmosphere increases its intensity by evaporating moisture and concentrating the pigment. Both these factors, increase in the total amount of pigment and concentration by evaporation, produce a progressive deepening of color in old cultures.

Even if the temporary conditions of cultivation are quite constant, the chromogenic power of an organism may be modified by its previous history. In thermal death-point observations we have found interesting cases of this sort. Some streaks made from broth cultures which had been exposed to a temperature of 50° or 55° were deeper in color than was the normal for the organism, but in most cases they were much lighter. Sometimes streaks made from a yellow or an orange chromogen after such treatment were almost colorless, although successive transfers generally restored the normal properties. Finally, we have noticed in our work spontaneous variations in chromogenesis such as have been recorded by Neumann (1897), Conn (1900), and Sullivan (1905). The latter authors note that on a plate sown from a single colony there may develop colonies varying appreciably in shade from which selections of the extremes will produce quite distinct types. Neumann records the sudden appearance of widely different strains, as sectors in old and carefully sealed stab cultures. We have observed both phenomena in our cultures, and are inclined to attribute the first, and, more doubtfully, the second, to variation rather than contamination.

In spite of all these facts it is clear that, as the cocci normally occur in nature, chromogenesis is one of their most distinct and significant differences. In any series of plates sown with washings from the skin four well-marked types—red, yellow, orange, and

white—are pretty certain to occur. We have therefore included chromogenesis as one of our routine tests. The variations due to past and present environment are, of course, easily excluded by the maintenance of constant conditions. Our stock cultures were in all cases kept on agar at 20°, and cultures for chromogenesis were grown on that medium, and at that temperature, for two weeks. In order to avoid the apparent differences due to the vigor of growth or to evaporation, a portion of the growth was removed on a loop needle and spread out on white drawing-paper with a rough surface. After drying at the room temperature, the color was compared with an arbitrary standard scheme.

The color chart used for matching these colors was devised after a very careful study of the colors actually found among the Coccaceæ, and includes nine hues ranging from white through lemon-yellow, light cadmium, medium cadmium, lemon-yellow and cadmium orange, red and cadmium orange, to two different combinations of red with lemon-yellow. We have used under each hue, nine different chromas, obtained by successively increasing washes of the hues on white paper. The hue in each case is recorded by a Roman numeral; the chroma, or number of wash, by an Arabic subscript.

Liquefaction of gelatin.—The liquefaction of gelatin, like the property of pigment production, has been shown to be subject to fluctuating variations. Conn (1900) was able by selection to obtain from a single culture of a milk coccus a rapidly liquefying form, and one with almost no peptonizing power. Smith (1900) records similar experiences with colon bacilli and forms of *B. proteus*. There appears to be little correlation between liquefaction and any other power, since it is so common in widely separated groups of bacteria to find organisms differing in this respect, while identical in all other properties.

In studying liquefaction we have determined only the amount of the action exerted by each organism. The shape of the liquefaction in the stab culture has been shown by Whipple (1902) to vary within the widest limits, with slight differences in the character of the medium, and the Committee on Standard Methods (1905) has omitted this character from its list.

For determining the amount of liquefaction we have used the

method suggested by Clark and Gage (1905), which consists in inoculating gelatin tubes of 10 mm. diameter by spreading a suspension of the culture over the surface. Liquefaction proceeds in a stratiform fashion, and its amount may be read off in centimeters. With such a method one may determine the rapidity of liquefaction either

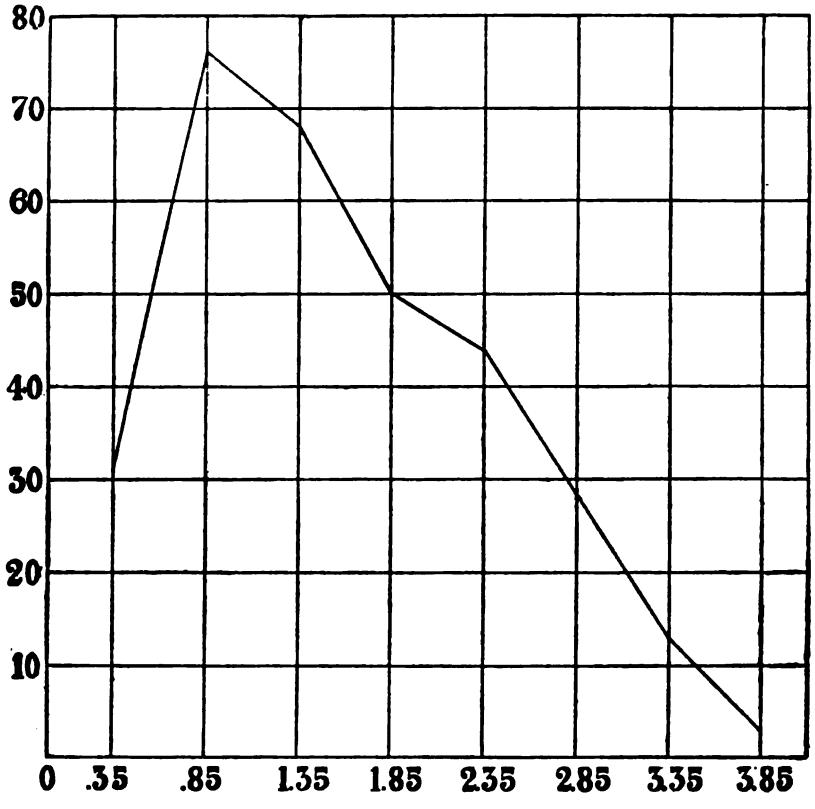

FIG. 4.—Liquefaction of gelatin by 314 cocci. Abscissæ, liquefaction in cm. Ordinates, number of cultures.

by the number of days required to reach a fixed point, or the final amount of liquefaction. In general, these two values are pretty closely correlated, but in a preliminary study we found that the final differences are somewhat sharper as well as easier to record. We have therefore adopted as our routine measure of liquefying power the depth of liquefaction after 30 days.

Supplementary tests.—Many other tests than those mentioned are sometimes used in bacterial diagnosis, but none have seemed suited to the present study. The questions of pathogenicity and agglutinative power are so shrouded in confusion as to be unpromising. Meyer (1902) considered serum reactions of diagnostic value among the streptococci, and Kolle and Otto (1902), and Otto (1903), obtained good results with the staphylococci. On the other hand, Aronson (1903), Fischer (1904), and Kerner (1905), after very thorough investigations, came to the conclusion that these properties among the streptococci are so erratic as to be quite valueless in systematic work. From a general survey of the literature of the group, it seems probable that the properties connected with infection and immunity are likely to be too easily modified to prove helpful in classification.

The test for liquefaction of starch is one which it seems logical to include with those which show the relation of an organism to gelatin and the sugars; and we made some experiments with the starch media introduced by Smith (1905). It appeared that certain cocci did exert an amylolytic action and the study of this character would probably prove of considerable interest. It has been omitted for the present, for lack of time.

III. RESULTS OF THE INVESTIGATION.

The characters observed and the terms in which they are recorded may be summarized as follows:

1. *Habitat.*—Recorded as 1 (diseased conditions); 2 (normal body); 3 (water); 4 (earth); or 5 (air). The significance of these various habitats has been more fully discussed above. It should be noted that Group 5 includes certain laboratory cultures whose origin was unknown.

2. *Grouping of cells and dimensions.*—Observed in stained preparations, made from 20° agar cultures less than five days old. Grouping recorded as 1 (packets present); or 2 (packets not present). Extreme dimensions recorded in micromillimeters to the nearest 10th.

3. *Relation to Gram stain.*—Observed on two occasions on 20° agar cultures less than five days old. Treated with anilin-oil-gentian-

violet for one and one-half minutes; Gram's solution, one and one-half minutes; 95 per cent alcohol, three minutes. Counterstained with Bismarck brown for one-half minute. Reaction recorded as – (decolorized in both tests); ± (stained once and decolorized once); or + (stained in both tests).

4. *Vigor of surface growth on agar streak after 14 days at 20°.*—Recorded as 1 (very faint); 2 (meager); 3 (good); 4 (abundant); or 5 (very heavy).

5. *Amount of acid produced in 2 per cent dextrose broth after 14 days at 20°.*—Determined by titration against $\frac{N}{20}$ NaCl in the cold with phenolphthalein as an indicator. Recorded value is the difference between inoculated tubes and sterile controls, expressed in cubic centimeters to nearest 10th.

6. *Amount of acid produced in 2 per cent lactose broth.*—Same conditions as under 5.

7. *Formation of nitrites in nitrate solution.*—Recorded value is the number of tubes giving positive test for nitrites out of a series of 10, grown for seven days at 20°.

8. *Formation of free ammonia in nitrate solution.*—Same method as under 7.

9. *Comparative growth after 14 days' growth on agar streak at 20° and 57°, respectively.*—Recorded as 1 (much more vigorous at 20°); 2 (more vigorous at 20°); 3 (equal); 4 (more vigorous at 37°); or 5 (much more vigorous at 37°).

10. *Chromogenesis.*—Hue and chroma of pigment produced on agar at 20° after 14 days, determined by comparison with color scheme as described later.

11. Depth in cm. of gelatin liquefaction in tube of 1 mm. diameter after 14 days at 20°.

It would be well to extend this series of tests by a study of the cell-grouping in broth, motility, fission on the agar block, fermentation of saccharose, effect of acid and alkaline media, and the thermal death-point.

1. HABITAT.

The distribution of the cultures isolated among the various habitats was as follows: (1) diseased conditions, 59; (2) normal body,

170; (3) water, 95; (4) earth, 67; (5) air, 109. It is probable that this deviates from a representative sampling of the cocci in nature by laying too great stress on the saprophytic forms. It is difficult to find cocci at all in earth and water, whereas they are present on the surfaces of the body in enormous numbers. The majority of this group appear to be parasitic or semiparasitic in habit. At the same time, the fairly equal weight given to the saprophytic forms helps to bring out the differences between the two main groups, those living in or on the animal body (1 and 2), and those living in the outer world (3, 4, and many of 5).

We have prepared tables to show the distribution of each character among various habitats, and the relations shown are so suggestive as to warrant rather full discussion. In Table 1 is given the correlation between habitat and cell-grouping, and it is at once evident that the sarcinæ occur in greater proportion outside than inside the body.

In this and succeeding tables the figures represent the number of cultures showing each combination of characters out of the 500 studied.

TABLE 1.

CORRELATION BETWEEN HABITAT AND CELL-GROUPING.

	Diseased Conditions	Normal Body	Water	Earth	Air
No. packets	44	145	45	33	78
Packets	15	25	50	34	31

Whereas packets are more abundant in earth and water, the other forms—chains, plates, and irregular groups without sarcinæ—make up two-thirds or more of the parasitic forms.

TABLE 2.

CORRELATION BETWEEN HABITAT AND GRAM STAIN.

Gram Stain	Diseased Conditions	Normal Body	Water	Earth	Air
−	14	12	46	37	36
±	17	50	29	18	32
+	28	108	20	12	41

The distribution of our cultures according to their relation to the Gram stain brings out a similar condition. The cultures giving

a consistent positive reaction make up far more than half the total among the parasitic forms from the first two habitats, and less than one-fourth of the forms from water and earth. The air cultures in almost all cases exhibit an intermediate relation, as would be expected, since they must contain forms from both sources. A positive reaction to the Gram stain is evidently closely correlated with life in and on the animal body.

TABLE 3.

CORRELATION BETWEEN HABITAT AND SURFACE GROWTH.

Surface Growth	Diseased Conditions	Normal Body	Water	Earth	Air
Very faint	0	16	3	0	0
Meager	1	15	2	2	1
Good	23	98	38	24	32
Abundant	27	34	38	31	60
Very heavy	8	7	14	10	16

The abundance of surface growth also varies with the habitat. Very faint and meager growths are fairly abundant in the forms from the surfaces of the body, as would be expected, since our culture media are unfavorable for the more strictly parasitic forms. On the other hand, a majority of the earth and water cocci show abundant or very heavy surface growths. The air forms are characterized by particularly abundant development, as would naturally be expected, since only the vigorous cells probably survive drying and dispersal through the air.

TABLE 4.

CORRELATION BETWEEN HABITAT AND THE FERMENTATION OF DEXTROSE BROTH.

Acid Production per Cent of Normal	Diseased Conditions	Normal Body	Water	Earth	Air
0.0 and alkaline	12	12	39	28	20
0.1–0.2	7	33	22	24	24
0.3–0.6	20	82	19	10	25
0.7–2.0	17	38	12	5	34
2.0 and over	3	5	3	0	6

TABLE 5.

CORRELATION BETWEEN HABITAT AND THE FERMENTATION OF LACTOSE BROTH.

Acid Production per Cent of Normal	Diseased Conditions	Normal Body	Water	Earth	Air
−0.2 and more alkaline	6	12	9	10	13
−0.1 and 0.0	23	37	62	42	40
0.1–0.4	15	64	16	11	32
0.5–1.4	11	49	5	4	19
1.5 and over	4	8	3	0	5

In examining Tables 4 and 5, which show the fermentative power of dextrose and lactose broth, the fundamental difference between the parasitic and saprophytic cocci is again made evident. In the first two groups, from the animal body, over two-thirds of the cultures produce more than 0.3 per cent of normal acid; while among the earth and water forms two-thirds of the organisms form less than this amount. With lactose the same law holds. Two-thirds of the cocci from the normal body produce acid in lactose, against less than one-third of the water and earth forms. The air cultures show an intermediate relation.

TABLE 6.

CORRELATION BETWEEN HABITAT AND REDUCTION OF NITRATES.

	Diseased Conditions	Normal Body	Water	Earth	Air
No reduction	44	147	75	44	72
Nitrites formed	10	19	13	10	18
Ammonia formed	7	7	8	13	30

The property of nitrate reduction does not appear to be related to habitat in any such direct way as the other characters studied. The air cocci, however, show a peculiarity of considerable interest, nitrite formation being common, and ammonia formation very common, among them.

TABLE 7.

CORRELATION BETWEEN HABITAT AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum	Diseased Conditions	Normal Body	Water	Earth	Air
20°	9	11	29	23	11
20° or 37°	36	112	56	42	89
37°	14	47	10	2	9

While a majority of the cultures studied grow indifferently at 20° or 37°, it appears from Table 7 that among the parasitic forms a fair proportion are favored by the body temperature, while more of the earth and water forms develop best at 20°. With regard to the optimum temperature for color formation, no definite relation with habitat appears, except as involved in the double relation between chromogenesis and habitat, and chromogenesis and the optimum temperature for color formation. These figures are therefore omitted.

TABLE 8.

CORRELATION BETWEEN HABITAT AND CHROMOGENESIS.

Chromogenesis	Diseased Conditions	Normal Body	Water	Earth	Air
White	4	17	5	1	13
Yellow	33	37	76	50	58
Orange	21	116	6	10	28
Red	1	0	8	6	10

Table 8 brings out some of the most definite relations yet considered, between habitat and chromogenesis. It is evident that the white and orange forms are largely parasitic, and the yellow and red forms as distinctively saprophytic. More than half of the white and more than two-thirds of the orange chromogens come from the first two habitats, while only one-third of the yellow forms have such an origin. The distribution of the red pigment-formers is even more notably saprophytic. Only one culture out of 25 occurred among the 229 cultures from the body.

TABLE 9.

CORRELATION BETWEEN HABITAT AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (Depth in cm.)	Diseased Conditions	Normal Body	Water	Earth	Air
0.0	13	68	43	26	36
0.1–1.5	24	36	42	30	45
1.6 and over	22	66	10	11	28

The table for habitat and gelatin liquefaction (Table 9) shows this property occurring among the earth and water forms to a less degree than among the parasitic cocci. This fact, and the fact that the parasitic forms are high acid-producers, as noted above, are of practical significance in connection with the bacteriological analysis of water. It has long been suspected that acid production and gelatin liquefaction were associated with intestinal organisms, and we have here a measure of the truth of this proposition, in the case of the cocci at least. From the food conditions which obtain in the alimentary tract, and to a less extent on the outer surfaces of the body, it is natural that these properties should be highly developed.

The forms from Habitat II (the surfaces of the normal body) group themselves most abundantly at two extremes, 68 being non-

liquefiers and 66 active liquefiers, while only 36 occupy the intermediate position, which is of most frequent occurrence in all the other habitats. It is probable that this may be accounted for by the presence, in Habitat II, of two distinct series—the white and colorless forms, which, as we shall see later, are non-liquefiers, and the orange forms, which peptonize strongly.

From a general survey of our habitat studies it is evident that the forms from the body exhibit quite different characteristics from those of the water and earth cocci. The parasitic forms generally react positively to the Gram stain, give only fair surface growths on the surface of artificial media, produce acid in dextrose and lactose, grow best at 37°, produce no pigment or a white or an orange pigment, and liquefy gelatin. The saprophytes, on the other hand, are more apt to occur in packets, to be Gram-negative, to grow abundantly on artificial media, best at 20°, to produce yellow and red pigments, and to exert little action on sugars and gelatin. The air cocci are generally intermediate in character between the two groups, but show special powers of nitrate reduction.

2. Grouping of Cells, and Dimensions.

The cocci, as noted above, were divided into two classes only, according to the character of the cell aggregates; 155 cultures showed the packets or sarcina-grouping, and 345 did not.

TABLE 10.

Correlation between Cell-Grouping and Gram Stain.

Gram Stain	Irregular Groups and Chains	Packets
−	75	70
±	98	48
+	172	37

We have pointed out above that packets are most abundant among the saprophytic cocci of the earth and water. Table 10 shows the relation between cell-grouping and the Gram stain, clearly indicating that the packets tend to be Gram-negative, while a majority of the other forms give a positive reaction.

Table 11 shows a distinct correlation between cell-grouping and the vigor of surface growth. A large majority of the non-packet-

forming organisms produce only very faint, meager, or good growths while a large majority of the sarcinæ form abundant or very heavy

TABLE 11.

CORRELATION BETWEEN CELL-GROUPING AND SURFACE GROWTH.

Surface Growth	Irregular Groups and Chains	Packets
Very faint	18	1
Meager	16	5
Good	169	46
Abundant	132	58
Very heavy	10	45

growths. The fact that the packet-forms flourish on artificial media should naturally result from their saprophytic origin.

TABLE 12.

CORRELATION BETWEEN CELL-GROUPING AND THE FERMENTATION OF DEXTROSE BROTH.

Acid Production (Per Cent Normal)	Irregular Groups and Chains	Packets
0 and under	58	53
0.1–0.2	56	54
0.3–0.6	128	27
0.7–2.0	87	20
2.0 and over	16	1

TABLE 13.

CORRELATION BETWEEN CELL-GROUPING AND THE FERMENTATION OF LACTOSE BROTH.

Acid Production (Per Cent Normal)	Irregular Groups and Chains	Packets
—0.2 and more alkaline	38	11
0.1 and 0.0	113	91
0.1–0.4	104	35
0.5–1.4	72	16
1.5 and over	18	2

A marked correlation between the packet-forming organisms (presumably saprophytic) and the fermentation of sugars is manifested in Tables 12 and 13. Taking Table 12 as an illustration, there will be found to be about 60 per cent of the packet-formers producing alkali, or, at most, fermenting dextrose but slightly (to 0.2)—almost none of the organisms occurring in the class of highest acid producers. Conversely, 70 per cent of the organisms which do not form packets produce acid from 0.3 up to the highest amount.

The power to reduce nitrates appears with about equal regularity in both our morphological groups.

TABLE 14.

CORRELATION BETWEEN CELL-GROUPING AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum Temperature	Irregular Groups and Chains	Packets
20°	40	43
20° or 37°	237	98
37°	68	14

A slight but distinct correlation appears between grouping and optimum temperature for growth. In each case most of the cultures grow at 20° or 37° indifferently; but a fair proportion of the more saprophytic sarcinæ show better development at 20°, while among the other forms more find an optimum at 37° than at 20°. With regard to the optimum temperature for color formation there is a slight difference, only two-fifths of the sarcinæ showing more chromogenesis at 20° than at 37°, against one-half of the other cultures. This, as we shall see later, is probably connected with a difference in chromogenesis.

TABLE 15.

CORRELATION BETWEEN CELL-GROUPING AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (cm.)	Irregular Groups and Chains	Packets
0.0	127	59
0.1–1.5 cm	110	67
1.6 and over	108	29

Table 15, for the relation of gelatin liquefaction to morphology, shows only that the highest grades of liquefaction are somewhat less numerous among the packets than in the other group.

The results obtained with regard to the size of the individual cell were much less suggestive than the facts concerning cell-grouping. Dimensions were measured in all cases on the stained specimens, and were recorded independently on at least two occasions. The attempt was made to note in each case the extreme diameters observed, and the values finally adopted represent the average between the recorded extremes. Individual cells ranged from 0.1 to 2.0 μ. With the packets it was found impossible to determine the maximum size of the single cell, on account of the frequent occurrence of small, recently formed packets which stained as a whole like one cell. We never felt certain that what appeared like a large cell was not

really a group of eight small ones. The sarcinæ in general showed quite small individual units, 0.1 or 0.2 μ as a rule, with no constant deviations. The packets are therefore entirely omitted from the consideration of dimensions.

The average sizes of the 345 cultures, not occurring in packets, are grouped in convenient classes in Table 16 and plotted in Fig. 1.

TABLE 16.

DIMENSIONS OF 345 COCCI.

Size, average, μ	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Number of cultures	22	82	113	60	40	12	7	6	1	2

The sizes of the cocci studied are evidently distributed on a fairly normal curve of frequency. The mode is at 0.3 μ and the curve is markedly skew, with infinite extension toward the larger sizes. The important practical point is that the forms measured appear to behave like a fairly homogeneous series varying about a single mode.

We have made tentative calculations of correlation between cell dimensions and other characters, with almost entirely negative result. The only property showing any relation is that of gelatin liquefaction.

TABLE 17.

CORRELATION BETWEEN CELL DIMENSIONS AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (cm.)	Maximum Size 0.3 μ and under	Maximum Size over 0.3 μ
0.0	47	78
0.1–1.5	65	47
1.6 and over	69	39

An appreciable inverse correlation is shown between the size of the cell and the rate of gelatin liquefaction, the smaller cocci liquefying most readily.

3. GRAM STAIN.

We have pointed out above that the reaction of the cocci to the anilin-oil-iodin stain is a variable character, many forms showing a positive reaction on one occasion and a negative reaction when next tested. Nevertheless the test, variable as it is, shows quite constant relations to other characteristics; and we feel convinced that among the cocci where all characters are more or less fluctuating any prop-

erty which on the average shows a definite correlation with other properties has systematic significance.

Of the cocci studied, 145 showed in two tests a Gram negative reaction on both occasions, and 209 two positive reactions, while 146 were once stained and once decolorized. Grouped thus in three divisions, we have seen that the positive reaction is characteristic of the parasitic forms, while saprophytic forms and packets tend to be Gram negative. With surface growth only an insignificant relation appears, the less richly growing forms showing a slightly higher proportion of positive reactions.

TABLE 18.

CORRELATION BETWEEN GRAM STAIN AND THE FERMENTATION OF DEXTROSE BROTH.

Acidity Produced (Per Cent Normal)	Gram Negative	Gram Variable	Gram Positive
0.0 and over	49	44	18
0.1–0.2	48	29	33
0.3–0.6	35	45	75
0.7–2.0	12	26	69
2.1 and over	1	2	14

TABLE 19.

CORRELATION BETWEEN GRAM STAIN AND THE FERMENTATION OF LACTOSE BROTH.

Acidity Produced (Per Cent Normal)	Gram Negative	Gram Variable	Gram Positive
−0.2 and more alkaline	20	14	15
−0.1 and 0.0	72	82	50
0.1–0.4	34	24	81
0.5–1.4	19	20	49
1.5 and over	0	6	14

The relation between the Gram reaction and the fermentation of carbohydrates is a surprisingly close one. Each line in Tables 18 and 19 showing the distribution of organisms among the grades of acidity forms a regular curve. In each case the mode of the Gram-negative cultures occurs at the neutral point, and that of the Gram-positive cultures at a moderately high acidity, with the doubtful cultures showing an intermediate relation.

TABLE 20.

CORRELATION BETWEEN GRAM STAIN AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum Temperature	Gram Negative	Gram Variable	Gram Positive
20°	34	29	20
20° or 37°	103	89	143
37°	8	28	46

With nitrate reduction and the optimum temperature for chromogenesis the Gram reaction shows no special relations. With optimum growth temperature, on the other hand, Table, 20 shows a distinct connection. As always, most of the cultures grow equally at both temperatures. Among the decolorized cultures, however, a fair proportion grow best at 20°, while with the positive forms 37° is most favorable. Such a relation would, of course, be expected from the generally saprophytic habit of the negative forms.

The liquefaction of gelatin does not show any distinct relation to the Gram reaction. On the whole, therefore, we may conclude that the cocci which decolorize by Gram are generally earth and water forms, which notably fail to ferment sugars, and which grow best at 20°. The marked correlation with the power of acid production, in the absence of other equally marked relations, seems to invite further study of the physiological basis of these properties.

4. SURFACE GROWTH.

The cocci studied were divided into five groups according to the vigor of surface growth on agar. The first group, of "very faint" growths, includes 19; the second group, of "meager" growths, includes 21 forms; "good" and "abundant" growths occur 215 and 190 times, respectively; and 55 cocci show "very heavy" growths.

TABLE 21.

CORRELATION BETWEEN SURFACE GROWTH AND THE FERMENTATION OF DEXTROSE BROTH.

Acidity Produced (Per Cent Normal)	Very Faint	Meager	Good	Abundant	Very Heavy
0.0 and alkaline	3	2	42	41	23
0.1–0.2	1	3	41	51	14
0.3–0.6	3	11	76	54	12
0.7–2.0	5	4	53	39	5
2.0 and over	7	1	3	5	1

TABLE 22.

CORRELATION BETWEEN SURFACE GROWTH AND THE FERMENTATION OF LACTOSE BROTH.

Acidity Produced (Per Cent Normal)	Very Faint	Meager	Good	Abundant	Very Heavy
−0.2 and more alkaline	2	3	18	22	5
−0.1 and 0.0	2	5	80	87	30
0.1–0.4	5	8	65	49	11
0.5–1.4	2	4	47	27	8
1.5 and over	8	1	5	5	1

We have seen above that the fainter growths are characteristic of the parasitic forms, and the heavier ones of the earth and water cocci. The heavier growths are more common among the packets than with other cell groupings.

In comparing the vigor of surface growth with the fermentation of carbohydrates, a distinct relation appears at the ends of the scale, although the bulk of the growth, under the headings "good" and "abundant," exhibit uniform characteristics. The "very faint" growths, which denote members of the genus Streptococcus, as previously defined, are associated with a maximum of acid production falling in the highest acidity class in each sugar table. On the other hand, the "very heavy" growths are mainly forms which fail to act on either sugar.

TABLE 23.

CORRELATION BETWEEN SURFACE GROWTH AND NITRATE REDUCTION.

	Very Faint	Meager	Good	Abundant	Very Heavy
No reduction	19	18	173	133	39
Nitrites formed	0	2	25	38	5
Ammonia formed	0	1	23	29	12

Surface growth and nitrate reduction show a suggestive relation. Among the very faint growths of the Streptococcus type no reduction occurs, and almost none among the "meager" forms. The "good" and "abundant" groups show an increasing proportion of reducing organisms, and the "very heavy" group shows many ammonia-formers and a fair proportion of nitrite production.

TABLE 24.

CORRELATION BETWEEN SURFACE GROWTH AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (Depth in cm.)	Very Faint	Meager	Good	Abundant	Very Heavy
0.0	19	14	63	80	10
0.1–1.5	0	6	84	62	25
1.6 and over	0	1	68	48	20

With gelatin liquefaction there exists the same group correlation manifest for the other characters. The "very faint" group shows not one liquefier, and the "meager" group very few, while the more vigorous forms exhibit a more even distribution.

In general, our study of surface growth brings out two distinct

groups of organisms. The first group, including the forms with faint or meager surface development, corresponds to the genus *Streptococcus* as defined above. It is sharply characterized by high acid production and the absence of gelatin liquefaction or nitrate reduction. Cocci of this type are characteristically parasitic, and very rarely show the sarcina grouping. On the other hand, the more vigorous forms are generally saprophytic, and frequently show packets. They ferment sugar slightly or not at all, and often reduce nitrates and liquefy gelatin.

5. FERMENTATION OF CARBOHYDRATES.

In measuring the amount of acid produced in dextrose and lactose broth, two check determinations were made in each case, and the figure finally recorded was the average of these two determinations. The correspondence between the two tubes was generally close. From 150 cases for each sugar we have calculated the probable error of a single observation, and find it only ± 0.043 for dextrose and ± 0.036 for lactose. Since our readings were only taken to 10ths of a c.c., it is evident that even a single determination would be sufficiently accurate for any long series.

The general results of the titrations are shown in Table 25 and in Fig. 2. It will be noticed that with both acids the organisms are ranged with fair regularity about a single mode. The majority of the cultures studied produce an acidity of 0.1–0.2 per cent normal in dextrose, and fail to ferment lactose at all. In either case a few cultures only show an alkaline reaction, and with lactose less than half of the organisms form acid, giving the curve for that sugar a very acute form. The curve for dextrose falls off much more slowly, and shows slight secondary modes at acidities of 0.5–0.6 per cent normal and 1.3–1.4 per cent normal. Finally, both curves show an extraordinary extension in the direction of the higher acidities. It will be noticed that for each sugar several of the highest reactions, ranging from 3 to nearly 10 per cent normal, have been omitted from the chart. We may fairly consider the fermentation of the two carbohydrates together, since, as shown in Table 26, they are very closely correlated. The amount of acid produced in lactose broth is almost always less than that in dextrose broth, but the two vary together.

TABLE 25.

ACID PRODUCTION IN SUGAR BROTHS.

Acidity Produced (Per Cent Normal)	Number of Cultures in Each Group							
	−0.9–0.8	−0.7–0.6	−0.5–0.4	−0.3–0.2	−0.1–0.0	0.1–0.2	0.3–0.4	0.5–0.6
Lactose	1	1	7	41	204	86	52	44
Dextrose	..	..	4	9	98	110	80	76

	0.7–0.8	0.9–1.0	1.1–1.2	1.3–1.4	1.5–1.6	1.7–1.8	1.9–2.0	2.1–2.2	2.3–2.4
Lactose	23	10	7	4	5	2	1	3	2
Dextrose	45	28	12	13	6	2	0	4	1

	2.5–2.6	2.7–2.8	2.9–3.0	3.1–3.2	3.3	4.0	4.3	4.6	4.7	5.9	8.2	8.6	9.7
Lactose	1	1	..	..	1	..	2	1	..	1	..	..	..
Dextrose	3	2	0	2	..	1	..	..	1	..	1	1	1

TABLE 26.

CORRELATION BETWEEN FERMENTATION OF DEXTROSE AND LACTOSE BROTHS.

Dextrose Acid Production (Per Cent Normal)	Lactose −0.2 and more alkaline	Lactose −0.1 and 0	Lactose 0.1–0.4	Lactose 0.5–1.4	Lactose 1.5 and over
0 and alkaline	19	72	15	4	1
0.1 and 0.2	11	57	29	13	0
0.3–0.6	11	47	55	40	1
0.7–2.0	9	26	36	29	6
2.0 and over	0	2	1	2	12

We have noted before that the power of carbohydrate fermentation is specially characteristic of the parasitic cocci, and of those which do not show the packet grouping. The high acidities are also correlated with a positive reaction to the Gram stain and with a faint surface growth on artificial media.

TABLE 27.

CORRELATION BETWEEN FERMENTATION OF DEXTROSE BROTH AND NITRATE REDUCTION.

Dextrose Acid Production (Per Cent Normal)	No Reduction	Nitrite Found	Ammonia Found
0 and alkaline	75	13	16
0.1 and 0.2	86	11	16
0.3–0.6	115	28	18
0.7–2.0	90	18	15
2.0 and over	16	0	0

Table 27, for the relation between nitrate reduction and acid formation in dextrose broth, shows only that the class of strong

acid-formers fail entirely to form nitrites and ammonia. A correlation table for lactose, which we have not thought it necessary to quote here, shows a similar relation. The high acid-formers, it may be remembered, belong to the genus *Streptococcus*, with its weak power of growth on artificial media.

Our tables of the correlation between carbohydrate fermentation and optimum temperature fail to show any striking coincidences. There is an appreciable tendency for the higher acid-formers to grow better at 37°, and for the alkaline or neutral forms to grow better at 20°; but we have not considered this important enough to warrant the reproduction of the tables.

TABLE 28.

CORRELATION BETWEEN GELATIN LIQUEFACTION AND FERMENTATION OF DEXTROSE BROTH.

Acid Production (Per Cent Normal)	Gelatin Not Liquefied	Gelatin Liquefied (0.1–1.5 cm.)	Gelatin Liquefied (1.6 cm. and over)
0 and alkaline.....	38	61	12
0.1 and 0.2.....	45	42	23
0.3–0.6.....	47	37	72
0.7–2.0.....	43	36	27
2.0 and over.....	13	1	3

TABLE 29.

CORRELATION BETWEEN GELATIN LIQUEFACTION AND FERMENTATION OF LACTOSE BROTH.

Acid Production (Per Cent Normal)	Gelatin Not Liquefied	Gelatin Liquefied (0.1–1.4 cm.)	Gelatin Liquefied (1.5 cm. and over)
–0.2 and more alkaline	20	19	11
–0.1 and 0.....	91	86	27
0.1–0.4.....	49	46	43
0.5–1.4.....	13	25	50
1.5 and over.....	13	1	6

The relation between the organisms which ferment the sugar broths and liquefy gelatin is shown in Tables 28 and 29. These tables may be considered together, as they reveal practically the same law. The relation between acid production and gelatin liquefaction is evidently a somewhat complex one. The forms which fail to ferment carbohydrates for the most part exhibit a moderate amount of liquefaction. Next comes a group of the moderate acid-producers which liquefy most actively. Finally, the highest acid-formers are mostly non-liquefiers. We shall get more light on these three groups when we come later to consider the classes of the cocci according to their chromogenesis.

To our conception of the non-acid-forming cocci as typically saprophytic organisms frequently occurring in packets and usually Gram-negative, we may add the property of moderate, but not very active, liquefaction of gelatin. The very high acid-producers are generally parasitic, do not show sarcinæ, stain by Gram, grow faintly on agar, and fail to reduce nitrates or liquefy gelatin. Between these two groups is a third type which forms a moderate amount of acid and produces the most active liquefaction of gelatin.

6. REDUCTION OF NITRATES.

As described above, the tests for nitrate reduction were made in parallel in 10 tubes, and a marked variation was found in the individual tubes, as shown in Table 30. This is perhaps to be expected, since the development of bacteria in such a nutrient medium as nitrate solution must be subject to many chance variations in the number and vigor of the organisms inoculated.

TABLE 30.

REDUCTION OF NITRATES.

Number of Tubes Showing Positive Tests	1	2	3	4	5	6	7	8	9	10
Nitrites	27	14	8	5	11	5	11	7	12	24
Ammonia	30	26	21	15	16	10	2	11	4	22

Table 30 shows a considerable number of cultures yielding positive results in one or two out of the 10 tubes tested, less in from four to seven of the tubes, and more again giving check results in all 10 tubes. In order to compare this property with others, it was necessary to distinguish between positive and negative cultures, and we have therefore considered the test to be positive when five or more of the tubes showed some reduction. The cultures then grouped themselves into three classes—one a large one, of those organisms which did not have the property of nitrate reduction, and the two smaller classes, in which were those which formed nitrites and those which formed ammonia.

Table 31 shows a somewhat surprising lack of correlation between the formation of the two reduction products for which we have made tests. Only 17 cultures showed both nitrites and ammonia in five

or more of the 10 tubes, while 48 cultures formed nitrites alone, and 53 cultures ammonia alone, according to the same standard. It seems improbable that in the latter case nitrites had been formed and entirely reduced to ammonia. We are inclined rather to conclude

TABLE 31.

CORRELATION BETWEEN NITRITE FORMATION AND AMMONIA FORMATION.

	Nitrites +	Nitrites −
Ammonia +	17	48
Ammonia −	53	382

that two different types of reduction exist, in one of which ammonia is produced directly.

As regards correlation with other properties, we have seen that the production of nitrites, and still more notably that of ammonia, is especially characteristic of the cocci isolated from the air. It is, of course, possible that this may be indirectly connected with the fact that forms which have survived drying and dispersal through the air must be particularly well adapted to conditions which obtain in the nitrate solution. A similar law is apparently manifest in the striking relation to vigor of surface growth. The power of forming both reduction products increases progressively with the richness of surface growth, being entirely absent in the "very faint" class. No relation appears between nitrate reduction and optimum temperature, and the only other correlation to be considered is that with gelatin liquefaction shown in Table 32.

TABLE 32.

CORRELATION BETWEEN NITRATE REDUCTION AND GELATIN LIQUEFACTION.

Gelatin Liquefaction (in cm.)	Nitrates Not Reduced	Nitrites Formed	Ammonia Formed
0.0	152	26	13
1.–1.5	131	27	25
1.6 and over	99	17	27

Table 32 shows the usual large proportion of organisms which do not exert nitrate reduction, but it may be noticed that some 30 per cent of the liquefiers reduce nitrates, against only 20 per cent of the non-liquefiers. Again, only 6 per cent of the non-liquefiers, against 16 per cent of the liquefiers, form ammonia.

7. OPTIMUM TEMPERATURE.

We have divided the cocci into five groups, according to their optimum growth temperature. Forty-one cultures gave "much better," and 42 "better," growth at 20°; 335 developed "equally" at both temperatures; 57 grew "better," and 25 "much better," at 37°.

We have classed together the first two and last two groups. In making the tables it was more convenient to have fewer groups, and quite as accurate, since the main distinctions (and those not very rigid) are shown in "better growth at 20°" or "better at 37°," and "equal" growth at both temperatures.

We have already noted the correlation between optimum temperature and habitat, the parasitic forms growing best at 37°, and the saprophytic forms at 20°, when any difference appears. The sarcinæ belong notably to the second class, as do the Gram-positive cultures. These are the only correlations which have so far appeared.

We have been somewhat surprised not to find special correlation between the optimum temperature for growth and that for color production; but no such correlation appears. With gelatin liquefaction also no definite relation appears.

We have observed also the effect of the body and room temperature upon color production, but without important results. Of the cocci studied, 69 showed a very much higher chromogenic power at 20° than at 37°; 169 showed more color, but not so much more, at the lower temperature; in 245 cases no difference appeared, while 13 cultures showed more, and 14 cultures much more, pigment at 37°. We have calculated correlation tables for all the various characters studied, but in no case did any constant relation appear, except, as noted later, in connection with the kind of chromogenesis.

8. CHROMOGENESIS.

As noted above, chromogenesis was determined by matching the pigment dried on white paper against a color chart prepared after a thorough study of the colors actually found among the cocci. This chart included nine hues, designated by Roman numerals, corresponding to the pigments noted below the figure. Under

each hue were nine different chromas, indicated by Arabic numerals, each figure indicating the number of washes of pure color added to obtain the particular chroma.

The distribution of the organisms studied under these different colors is indicated in Fig. 3, where the vertical columns indicate the hues from I (white), through the yellows (II–IV), the oranges (V and VI) to the reds (VII–IX), and the horizontal columns the successive chromas.

On inspection of this chart, bearing in mind the colors signified, there appear at once four modes – one occurring in each chief color.

That for the white falls at I_1, for the yellows at IV_3, for the oranges at VI_8, and for the reds at VII_8. These are not, of course, the points of intensest color, but of the most concentrated distribution. The evident clustering of the individuals around a mode, and the consequent falling-away of the numbers between the modes, suggest a variation from an ancestral center. Like most living things governed by an evolutionary law of gradual change, the hues grade so gently into each other that the exact placing of lines of division must be arbitrary. We have, however, assumed four divisions as a basis for our work, and separated them at the lowest points between the modes, as shown by the heavy black lines in the chart, which divide the group of bacteria producing a white pigment from that which produces a yellow, the yellow from the orange, and the orange from the red. The striking correlations obtained between chromogenesis and other properties have convinced us that this division was a sound and natural one. It should be noted, however, that the division of the "white" chromogens includes two subgroups—the true white pigment-formers and the forms which produce such a faint surface growth that no distinct color is apparent.

We have omitted the consideration of chromogenesis from our correlation tables, except that for habitat, preferring to consider all chromogenic relations under one head. It will appear, on inspection of the following tables, that this character is really the key by which most of the other correlations may be explained, and is perhaps the most important single factor in the systematic grouping of the Coccaceæ.

It was shown under "Habitat" that the white and orange chromo-

gens were chiefly parasites, the yellow and red chromogens chiefly saprophytic forms. The same distinction is shown in Table 33 with regard to cell-grouping. The white and orange cocci only rarely,

TABLE 33.

CORRELATION BETWEEN CHROMOGENESIS AND CELL-GROUPING.

Cell-Grouping	White	Yellow	Orange	Red
Irregular Groups and Chains	33	134	163	15
Packets	7	120	18	10

the latter very rarely, show packets. The yellow and red forms, on the other hand, show the sarcinæ-grouping almost half the time.

TABLE 34.

CORRELATION BETWEEN CHROMOGENESIS AND GRAM STAIN.

Gram Stain	White	Yellow	Orange	Red
−	6	109	15	15
±	9	84	46	7
+	25	61	120	3

The reaction to the Gram stain exhibits a still more perfect correlation. Among the whites and oranges (the parasitic forms) positive Gram reactions predominate, and negative ones are rare. Among the saprophytic yellows and reds conditions are symmetrically reversed.

TABLE 35.

CORRELATION BETWEEN CHROMOGENESIS AND SURFACE GROWTH.

Surface Growth	White	Yellow	Orange	Red
Very faint	14	3	2	0
Meager	3	6	12	0
Good	7	100	107	1
Abundant	13	95	58	24
Very heavy	3	50	2	0

A comparison of the general vigor of growth shows that each color has its own relation. Among the white forms, two maxima appear, one under "very faint" growth and one under "abundant" growth. This is because this group is a compound one, including forms which give a really white growth abundant in amount, and the feebly growing streptococci which are classed here, although they produce no pigment at all. The yellow and orange chromo-

gens show maxima under the "good" growth, almost all the "very abundant" growths belonging to the former class. The red forms are almost all of one type—the "abundant."

TABLE 36.

CORRELATION BETWEEN CHROMOGENESIS AND DEXTROSE FERMENTATION.

Acid Produced (Per Cent Normal)	White	Yellow	Orange	Red
0.0 and alkaline	5	94	7	5
0.1–0.2	7	72	24	7
0.3–0.6	5	50	92	9
0.7–2.0	15	35	53	3
Over 2.0	8	3	5	1

TABLE 37.

CORRELATION BETWEEN CHROMOGENESIS AND LACTOSE FERMENTATION.

Acid Produced (Per Cent Normal)	White	Yellow	Orange	Red
–0.2 and more alkaline	3	33	9	5
–0.1 and 0.0	8	141	39	16
0.1–0.4	12	59	64	3
0.5–1.4	6	18	63	1
1.5 and over	11	3	6	0

The correlations between chromogenesis and the fermentation of the sugars are singularly perfect. The white forms in each case show two maxima, one corresponding to the true white chromogens, the second, at a higher acidity, to the colorless streptococci. The latter include a majority of the strongest acid-producers in each case. The other types show for each sugar a regular and characteristic curve, the elements from which the complex curve in Fig. 2 is made. The yellow forms show for each sugar a mode at the neutral point. The orange chromogens, on the other hand, are most abundant at an intermediate grade of acidity, most of them producing 0.3–0.6 per cent acidity in dextrose broth, and 0.1–0.4 per cent in lactose broth. The red forms show the same relation as the orange forms toward dextrose, while in lactose broth they resemble the yellow chromogens, producing in most cases no change of reaction.

TABLE 38.

CORRELATION BETWEEN CHROMOGENESIS AND NITRATE REDUCTION.

	White	Yellow	Orange	Red
No reduction	35	197	137	13
Nitrites produced	3	30	25	12
Ammonia produced	2	37	26	0

With regard to the reduction of nitrates, the white and colorless forms show generally negative results. Nitrites are produced by one in 10 of the yellows, a slightly higher fraction of the orange forms, and by half the red-pigment-producers. Ammonia production, on the other hand, appears in one in eight of the yellows, one in 10 of the orange forms, and not at all among the reds.

TABLE 39.

CORRELATION BETWEEN CHROMOGENESIS AND OPTIMUM TEMPERATURE FOR GROWTH.

Optimum Temperature	White	Yellow	Orange	Red
20°	4	66	13	0
20° or 37°	28	156	126	25
37°	8	32	42	0

Excluding the majority of forms which grow equally at either temperature, it appears that, among the white and orange forms, most of those which exhibit any preference grow best at 37°, while among the yellows 20° is more often the optimum. These results accord with the habitats, respectively parasitic and saprophytic, of the two classes.

TABLE 40.

CORRELATION BETWEEN CHROMOGENESIS AND OPTIMUM TEMPERATURE FOR COLOR FORMATION.

Color Production	White	Yellow	Orange	Red
Better at 20°	2	90	125	21
Equal at 20° and 37°	38	164	56	4

It appears from Table 40 that temperature differences affect the production of orange pigment much more than that of yellow and that the body temperature interferes with red chromogenesis most of all.

TABLE 41.

CORRELATION BETWEEN CHROMOGENESIS AND GELATIN LIQUEFACTION.

Gelatin Liquefaction, cm.	White	Yellow	Orange	Red
0.0	27	83	55	21
0.1–1.5	8	126	39	4
1.6 and over	5	45	87	0

The liquefaction of gelatin presents another close correlation with pigment production. The white and red forms are almost all non-liquefiers, the yellow cocci show a maximum among the

moderate liquefiers, and the orange chromogens exhibit the peptonizing power to a high degree.

To sum up, the cocci show four (or five) distinct groups according to their pigment production, each group being marked by a number of other correlated characters. The "white" forms rarely show packets, generally stain by Gram, fail to reduce nitrates, grow well at 37°, and usually fail to liquefy gelatin. They include two subgroups—the feebly growing, strongly acid-producing forms, which are really colorless, not white, and the white-pigment-producers, which grow abundantly and produce only a slight amount of acid. The "yellow" chromogens frequently show packets, are usually Gram-negative, give a good to a very heavy surface growth, produce little or no acid, occasionally form nitrites or ammonia in nitrate solution, grow well at 20°, and show a moderate liquefaction of gelatin. The "orange"-pigment-formers are very rarely packets, stain well by Gram, form good surface growths, produce a moderate acidity in sugar broth, occasionally reduce nitrates to nitrites or ammonia, grow well, but with poor pigment production, at 37°, and generally produce a considerable liquefaction of gelatin. The red-pigment-producers are often packets, generally Gram-negative, grow abundantly, ferment dextrose but not lactose, form nitrites, but not ammonia, in nitrate solution, grow well at 20° or 37°, producing less pigment in the latter case, and generally fail to liquefy gelatin.

9. GELATIN LIQUEFACTION.

In the routine determination of gelatin liquefaction we have used only one tube for each culture. Duplicate determinations were made on 79 cultures, from which it appeared that the probable error of a single observation is only ±0.12 cm.; so that our method was sufficiently accurate.

Of the cultures observed, 186 failed to liquefy gelatin, and the distribution of the other 314 cultures according to the amount of

TABLE 42.

GELATIN LIQUEFACTION.

Depth in cm.	0.1–0.5	0.6–1	1.1–1.5	1.6–2.0	2.1–2.5	2.6–3.0	3.1–3.5	over 3.5
Number of cultures	33	76	68	48	44	29	13	3

liquefaction after four weeks is shown in Table 42. Fig. 4 shows graphically the skew curve plotted from these data.

There is, as would be expected, a gradual falling-away toward the highest amounts of liquefaction, and the abrupt downward falling of the curve toward the non-liquefiers at 0 is extremely significant as indicating a sharp difference between the two groups. If it had been practicable to plot the non-liquefiers on this figure, the curve would have gone up at an acute angle more than twice as high as that of the mode of the liquefiers. This angle divides with more than usual definiteness those organisms which liquefy from those which do not liquefy gelatin.

The correlations of gelatin liquefaction with other properties have been already considered. We have found that a high peptonizing power is rare among the earth and water cocci and the sarcinæ. It is most frequently associated with the smaller individual cells among the non-packet-formers. It is absent or very rare in the cocci which show only faint surface growths. Finally, it appears that the white, colorless forms which produce high acidities, as well as the red chromogens, are non-liquefiers. The yellow cocci which produce little acid are moderately active liquefiers, and the orange forms with a moderate acid production show the highest peptonizing power.

IV. CONCLUSIONS FROM THE INVESTIGATION.

1. FOUNDATION OF SUBFAMILIES AND GENERA AMONG THE COCCI.

The extreme variability of the cocci has appeared with great clearness in the present study. Almost every one of the characters measured shows a wide range of fluctuations. In view of the general laws of variation, the absence of sexual reproduction, and the susceptibility of the bacteria to the direct influence of the environment, this is precisely what should be expected. It makes it, however, clearly impossible to draw sharp and arbitrary lines for any single character by which individual organisms can be naturally classified.

If, on the other hand, we examine a series of individuals with the idea of discerning central types about which they vary, the problem begins to solve itself, since such types are easily apparent. Certain organisms tend to show the packet grouping—some invariably

in every aggregate, some less constantly. Other organisms never show packets, or only very rarely. Some cocci always stain, and some always decolorize, by Gram, while intermediate forms tend more or less strongly toward either type. In surface growth two distinct types, the faint to meager and the abundant to very heavy, are manifest. In acid production there appear to be three centers of distribution corresponding to organisms which fail to ferment, those which ferment slightly, and those which produce large amounts of acid. In relation to nitrate reduction, three types appear, according as the cocci fail to reduce, or form nitrites or ammonia. On gelatin the organisms studied group themselves either as liquefiers or as non-liquefiers, and in color production four distinct centers appear, in which the pigment is white, yellow, orange, or red.

Our estimate of the value of these type-centers is greatly increased when we find that the central points for the different characters do not vary independently, but are correlated together to a remarkable degree. Again, we should expect, and we actually find, in some cases, the correlation of single characters varying, those properties generally correlated appearing in certain organisms in exceptional combinations. If, however, we consider, not the single character—not the individual organism—but the aggregate of the correlations of various properties as manifested in a considerable series of individuals, certain well-defined systematic units appear, marked by the association of a number of independent characteristics. Such an association can be explained only on the ground of relationship, and the types marked by the simultaneous occurrence of a number of properties may rightly be taken as the centers from which other, more aberrant individuals have varied.

The fact that correlation exists shows that, on the average, the fluctuations of these characters do not occur independently, but are so closely bound up with those of other properties as to vary together with them. This may be because the selective action of the environment produces a parallel change in each, or because the two characters are so closely bound together, in the physiological balance of the organism, that a change in one leads to a corresponding variation in the other. In either event it is clear that the larger systematic units (families or genera) must be marked by these pro-

found modifications of the whole center of gravity of the organism, and the smaller groups by those characters which, though perhaps showing sharper individual differences, vary by themselves without affecting any other properties.

Our object therefore has been, not to establish arbitrary boundary lines, but to discover existing natural types distinguished by the association of independent characters. In such a task it is obvious that those characters are most important which show the most marked correlations. What these characters are must be determined by study in each particular group. Chromogenesis or gelatin liquefaction may be of generic value in one family, or may mark only varieties in another, as it is, or is not, correlated with a number of other properties. In the Coccaceæ, for example, the liquefaction of gelatin and the reduction of nitrates appear, when judged by this standard, to be of less importance than most of the properties studied. In some cases they appear to be significant, but, in most of the groups indicated, liquefying and non-liquefying forms, and reducing and non-reducing forms, run parallel. Distinctions based on such a single character alone may have specific, but certainly not generic, value. On the other hand, we have been somewhat surprised to find that such apparently fluctuating characters as chromogenesis and the reaction to the Gram stain are strongly correlated with a number of other properties.

A general survey of the whole field of variation among the Coccaceæ indicates clearly the existence of two main sets of correlated characters, corresponding to the subfamilies which we have suggested in a previous communication (Winslow and Rogers, 1905). Habitat, morphology, staining reactions, surface growth, acid production, optimum temperature, and chromogenesis, all vary simultaneously in one or the other of two directions, defining the two subfamilies Paracoccaceæ and Metacoccaceæ. The first group, comprising most of the forms from the body, shows, as a rule, chains and irregular cell-grouping, stains by Gram, yields a meager or only fair surface growth, forms acid in carbohydrates, and produces no pigment, or a white or an orange one. The other group, from earth and water for the most part, often shows packets, decolorizes by Gram, grows well on artificial media, fails to ferment carbohydrates, and produces a

yellow or red pigment. It must always be remembered that each character may occasionally be found in the group where it usually does not occur; but the association of these properties in the vast majority of cases is very strong. We desire to extend our earlier definitions of the two subfamilies by including the Gram reaction and chromogenesis; and the subfamilies as thus modified will be defined at the end of this communication. It is a striking fact that these two chief divisions among the Coccaceæ correspond to the two markedly different environments which exist in nature, the body of higher organisms, and the outer world. A close correspondence with environmental conditions should naturally be expected among such simple asexual organisms as the bacteria, and it increases our confidence in the reality of the groups established below to find each of them localized so sharply in one or other of the two main environments.

Under the subfamilies we find a second grade of group-individuality, marked by the association of a smaller number of characters than the subfamilies, but still defined by the correlation of several independent properties. Here morphology, surface growth, and chromogenesis appear to be of greatest importance, acid production, gelatin liquefaction, and nitrate reduction having special significance in certain cases. Five distinct types have appeared with considerable clearness in the present study. It must be remembered that the fundamental correlations which revealed these groups were derived in an entirely impersonal way by measurements, made on each character independently, generally by different observers, and always without knowledge of the identity of the organism. When individual races are considered, it is possible, by transferring a few cultures on the border-line in a single character, to show that the correlations are really closer than have appeared above.

By this process we have attempted to group our 500 cultures under the five subdivisions suggested by the correlation tables, and have found the results so satisfactory as to confirm our confidence in their reality as natural groups. It seems to us that these groups are of such importance as to deserve generic rank. Within each there is ample room for the establishment of such a reasonable number of species as detailed study may warrant. Good genera must first be recognized, however. It is time that bacteriologists

were relieved of such vast and unwieldy and meaningless genera as now burden the science.

The first of these groups centers about a type of organism characterized by the following properties: it is parasitic in habit and grows in irregular groups, often in chains, never in packets; it stains by Gram; it grows in a thin film on the surface of agar; it ferments both lactose and dextrose with the production of a large amount of acid; it fails to reduce nitrates or liquefy gelatin, grows best at 37°, and forms no appreciable amount of pigment. This corresponds to the genus *Streptococcus* (Billroth) W. and R., as previously characterized. We desire to add to our previous conception of the group the positive reaction to the Gram stain and the general failure to act on gelatin or nitrates. It must always be remembered that this genus is defined, not on morphology alone, although its members generally do show long chains in broth, but by the general complex of all its characters. Individual cultures vary from the type in some respects, as must all aggregates of organisms composed of such varying stuff as living protoplasm.

Of our 500 cultures 18 fall into the genus *Streptococcus*. All show the typical morphology (groups and long and short chains) and typical surface growth. None liquefy gelatin or reduce nitrates. Of our 18 cultures, 15 were from the body and three from polluted water. In relation to the Gram stain, 11 cultures showed positive tests, on both trials, and only two a negative test, five being variable. Of the 18 cultures, nine showed very high acidities, over 2 per cent normal, in both acids, some ranging as high as 8 to 9 per cent. The average value for the whole genus is, for dextrose 2.6 per cent, and for lactose 1.7 per cent. It is interesting to notice that those cultures which yield lower acidities are also the ones which give the negative or variable Gram reactions. Our forms therefore seem to fall into two species, 10 of them belonging to the *Str. erysipelatos*, showing the very high acidities and the positive Gram reaction; the other eight differing in both these characters.

The second of our five groups is marked by a correlation of characters, of which the most obvious is the production of an orange pigment. In our previous communication we were unable to distinguish, from the literature alone, any sharp line between the orange

and yellow chromogens, and included them both under the genus *Micrococcus*. Fig. 3 makes it clear, however, that two distinct centers of variation exist, one in the orange and one in the yellow, and our correlation tables show that the two types of organisms are so radically different in every character as to demand their separation into distinct genera. Furthermore, it is evident that the orange chromogens belong with the parasitic Paracoccaceæ, and the yellow forms with the Metacoccaceæ. Nothing could show more clearly how necessary it is to make a comparative study of a large series of organisms in order to discern the true relationships of the bacteria.

For this new genus we suggest the name *Aurococcus*, as indicating the orange color, which is its most obvious characteristic. Its type-form is found on or in the plant or animal body. It occurs in groups and short chains, stains by Gram, and produces a good, but not heavy, surface growth of an orange color. It ferments dextrose and lactose, producing an acidity generally between 0.5 and 1. It grows well, but produces less pigment at 37°. It may or may not reduce nitrates and liquefy gelatin. When it does liquefy gelatin, it does so rather actively.

Of the 158 cultures in this group, all show a good, but not very abundant, growth of an orange color; 116 were obtained from the body and 30 from the air, only 12 having a saprophytic origin; 147 show groups and short chains, but no packets; and 11 occasionally give the sarcina grouping. Of the 158 cultures, 107 show a positive Gram reaction, and only nine a consistently negative one. The average acidity in dextrose for the whole group is 0.7 per cent normal, and for lactose 0.4 per cent normal. Of the 158 cultures only six form less than 0.2 per cent acid, and 17 more than 1 per cent acid, in dextrose. In lactose there is more variation; 53 cultures give less than 0.2 per cent, and 11 more than 1 per cent, acid. Of the cultures, 31 reduce nitrates, and 102 liquefy gelatin to an average depth of 2.2 cm.—a very high value; while 56 organisms fail to liquefy. The type-form of this genus is the commonest pyogenic organism, the *M. aureus* of Rosenbach. The non-liquefying forms, those which reduce nitrates, and those which produce more or less acid than is common in the genus, may later be set up as separate species.

The third of our types, like the second, has not previously been distinguished from the genus *Micrococcus;* it appears, however, to show its own definite individuality, and to belong with the Paracoccaceæ, although it approaches the saprophytic cocci in certain characters. We suggest the name *Albococcus* for this genus, which includes those organisms of which *M. pyogenes* (Ros.) Mig. is a type. They produce a more vigorous surface growth than the streptococci, with a clear white pigment, and ferment carbohydrates, producing a fair amount of acid. They are also distinguished from the Metacoccaceæ by the general tendency of their morphology and staining reactions, and by their habitat. In our series we have 23 cultures of this type. All without exception were obtained from the body or from the air, none from water or earth. All without exception show a good surface growth, white pigment, and division into groups and rarely chains, but never packets. Sixteen were uniformly Gram positive and only two uniformly Gram negative. The average acidity in dextrose broth was 0.7 per cent normal, and in lactose broth 0.5 per cent normal. Only three cultures showed an acidity lower than 0.2 per cent, and only one culture an acidity over 1.5 per cent in dextrose. Lactose results, as usual, were more variable, nine cultures falling below 0.2 per cent acid, and one above 1.5 per cent. Nitrates were reduced by three cultures and gelatin liquefied by 14. The four species which we have previously called *M. pyogenes* (Ros.) Mig., *M. rhenanus* Mig., *M. candicans* Flügge, and *M. canescens* Mig., should belong to this new genus, being distinguished, as before, by their relation to acid and gelatin. The reduction of nitrates may furnish a basis for the establishment of other species.

The fourth of the general groups which have appeared in this study is the group of the yellow pigment formers, of which *M. luteus* and *Sarcina ventriculi* are typical—a group which differs in almost all its properties from those previously considered. Organisms of this type are found mainly in earth and water rather than on or in the animal body. They give abundant, to very heavy, growths of a yellow color. They frequently occur in packets, generally decolorize by Gram, and fail to ferment sugars or ferment them only slightly. They may or may not liquefy gelatin or reduce nitrates.

Of our cultures 262 fall under this head, 195 of them coming from water, earth, or air, and only 64 from the body; 200 are uniformly or at times Gram negative, and only 62 uniformly Gram positive. The average acidity produced in dextrose broth is only 0.2 per cent normal, and in lactose broth 0.1 per cent normal. Of the 262 cultures only 33 give over 0.5 per cent acid in dextrose broth, and only 7 over 0.5 per cent acid in lactose broth; 59 of the cultures reduce nitrates; 85 fail to act on gelatin, and 177 liquefy it, producing an average liquefaction of 1.2 cm., scarcely more than half the value in the genus *Aurococcus*.

This group divides itself, according to cell-grouping, into two nearly equal divisions—those which form packets and those which do not; 136 belong to the former class, and 126 to the latter. In habitat, in Gram staining, and in relation to carbohydrates and gelatin both classes are entirely parallel. The genera *Micrococcus* and *Sarcina* are, however, so firmly established in common usage that it would require very strong evidence of identity to warrant dropping either of them. It seems best, therefore, to recognize the single character of cell-grouping as having generic value in this case, otherwise defining the two genera by the same characteristics. Under *Micrococcus* will come *M. orbicularis* Ravenel, *M. luteus* (Schröter) Cohn, and *M. ochraceus* Rosenthal; under *Sarcina*, *St. subflava* Ravenel and *S. ventriculi* Goodsir.

The fifth and last of our general types includes the sharply marked one of the red chromogens. These are entirely saprophytic forms which produce abundant surface growths of a red color. They may or may not show packets, are generally Gram negative, very rarely liquefy gelatin or ferment carbohydrates, and frequently reduce nitrates to nitrites, but apparently not to ammonia. This is the first case in which we have found the action upon nitrates markedly correlated with other characters.

Twenty-five of our cultures fall under this general type. All but one come from earth, water, or air. Only three are uniformly positive to Gram, while 15 are uniformly negative. Four of the 25 cultures liquefy gelatin, and 14 reduce nitrates to nitrites. The average acidity in dextrose broth is 0.4 per cent normal, and in lactose the average reaction is neutral. One culture in lactose and four in dextrose show an acidity over 0.5 per cent.

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Here again the packets (10 in number) and the other forms (15 in number) are exactly parallel. Both show the same range of acidities and the same peculiar relation to nitrate reduction. It seems quite clear that in this case the single character of packet formation ought not to be made the basis of a distinct genus. We have recognized among the yellow forms the two genera *Micrococcus* and *Sarcina* out of deference to custom, which must always play an important part in terminology. In separating the red forms from these two old genera, however, it seems an unreasonable recognition of a false distinction to form two new ones on the single character of cell-grouping alone. We desire, therefore, to include all the red-pigment forms, characterized by the properties noted above, under one new genus, to be called *Rhodococcus*. *M. cinabareus* Flügge, *S. rosacea* Lindner, and *S. incarnata* Gruber will all belong here. Fourteen doubtful cultures are for the present omitted from this generic classification.

It remains only to summarize the characteristics of the six genera studied in this investigation in tabular form, and then to present a systematic statement of the main divisions of the Coccaceæ. It must be remembered that *Diplococcus* and *Ascococcus* have not been included in the present research and are defined solely from the the literature.

TABLE 43.

CHARACTERS OF CERTAIN GENERA OF THE COCCACEÆ.

Genus	Habitat (Per Cent Parasitic Forms*)	Cell-Grouping (Per Cent of Packet-Formers)	Gram-Stain (Per Cent of + Results)	Surface Growth	Average Acidity in Dextrose (Per Cent Normal)	Average Acidity in Lactose (Per Cent Normal)	Nitrate Reduction (Per Cent of Reducers)	Chromogenesis	Gelatin (Per Cent of Liquefiers	Liquefaction (Average Liquefaction cm.)
Streptococcus	83	0	61	Faint	2.6	1.7	0		0	...
Aurococcus	76	7	68	Good	0.7	0.4	21	Orange.	65	2.2
Albococcus	48	0	70	Abundant	0.7	0.5	13	White..	61	1.1
Micrococcus	27	0	25	Good to Abun.	0.3	0.1	27	Yellow.	68	1.2
Sarcina	22	100	23	Good to Abun.	0.2	0.1	18	Yellow.	67	1.2
Rhodococcus	4	40	12	Abundant	0.4	0.0	56	Red...	16	0.7

* Body alone; air, source of many others. In *Albococcus* none from water or earth.

2. SYSTEMATIC SUMMARY.

Family Coccaceæ: Vegetative cells spherical.

Subfamily 1 Paracoccaceæ (Winslow and Rogers): Parasites (thriving only or best, on or in, the plant and animal body). Thrive

well under anaërobic conditions. Many forms fail to grow on artificial media; none produce very abundant surface growths. Planes of fission often parallel, producing pairs, or short or long chains, never packets. Generally stain by Gram. Produce acid in dextrose and lactose broth. Pigment, if any, white or orange.

Genus 1, *Diplococcus* (Weichselbaum): Strict parasites. Not growing, or growing very poorly, on artificial media. Cells normally in pairs surrounded by a capsule. Includes *D. pneumoniae* Weich, *D. Weichselbaumii* Trev., and *D. gonorrheae* Neisser.

Genus 2. *Streptococcus* (Billroth): Parasites (see above). Cells normally in short or long chains (under unfavorable cultural conditions, sometimes in pairs and small groups, never in large packets). Generally stain by Gram. On agar streak effused, translucent growth, often with isolated colonies. In stab culture, little surface growth. Sugars fermented with formation of large amount of acid. Generally fail to liquefy gelatin or reduce nitrates. Includes *S. erysipelatos* Fehleisen.

Genus 3, *Aurococcus*, new genus: Parasites (see above). Cells in groups and short chains, very rarely in packets. Generally stain by Gram. On agar streak good growth of orange color. Sugars fermented with formation of small amount of acid. Gelatin often liquefied, very actively. May or may not reduce nitrates. Includes *A. aureus* (Rosenbach).

Genus 4, *Albococcus*, new genus: Parasites (see above). Cells in groups and short chains (never in packets). Generally stain by Gram. Growth on agar streak abundant and porcelain white in color. Sugars fermented with production of a slight amount of acid. Gelatin liquefaction and nitrate reduction may or may not occur. Includes *A. pyogenes* (Rosenbach), *A. rhenanus* (Migula), *A. candicans* (Flügge), and *A. canescens* (Migula).

Subfamily 2, Metacoccaceæ (W. and R.): Facultative parasites or saprophytes. Thrive best under aërobic conditions. Grow well on artificial media, producing abundant surface growths. Planes of fission often at right angles; cell aggregates in groups, packets, or zoöglea masses. Generally decolorize by Gram. Pigment, yellow or red.

Genus 5, *Micrococcus* (Hallier): Facultative parasites or sapro-

phytes. Cells in plates or irregular masses (never in long chains or packets). Generally decolorize by Gram. Growth on agar abundant with formation of yellow pigment. Dextrose broth slightly acid, lactose broth generally neutral. Gelatin frequently liquefied. Nitrates may or may not be reduced. Includes *M. orbicularis* Ravenel, *M. luteus* (Schröter) Cohn, and *M. ochraceus* Rosenthal.

Genus 6, *Sarcina* (Goodsir): Exactly like *Micrococcus*, except that division occurs under favorable conditions, in three planes, producing regular packets. Includes *S. ventriculi* Goodsir, *S. aurantiaca* Flügge, *S. subflava* Ravenel, *S. tetragena* (Mendoza) Mig.

Genus 7, *Rhodococcus*, new genus: Saprophytes. Cells in groups or regular packets. Generally decolorize by Gram. Growth on agar abundant, with formation of red pigment. Dextrose broth slightly acid, lactose broth neutral. Gelatin rarely liquefied. Nitrates generally reduced to nitrites, but not to ammonia. Includes *R. cinnabareus*, Flügge, *R. roseus* Flügge, *R. fulvus* Cohn, *R. agilis* (Ali Cohen), *R. rosaceus* Lindner, and *R. incarnatus* Gruber.

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THE OCCURRENCE OF ORGANISMS OF SANITARY SIGNIFICANCE ON GRAINS.*

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IN the development of bacteriology as applied to the sanitary investigations of water supply, food supply, and sewage disposal, the colon bacillus (*B. coli*) and certain streptococci (*Strept. pyogenes*) have been regarded as of extraordinary significance. This has been especially true of *B. coli*, which has been studied unceasingly and by a large number of investigators almost ever since its discovery by Emmerich in 1885. Having been early shown to be a constant inhabitant of the human intestine, and present there in vast numbers, it is not surprising that it has been regarded as of great sanitary significance, and hence one of the most important of bacteria.

The streptococci here to be considered have received less attention, as they were more recently discovered, but as they have been proved to be present in sewage, sewage effluents, and polluted waters, and in the soil which receives the wastes of animal life, these too have been regarded as characteristic of pollution, and their detection has served as a striking confirmation of the evidence offered by the finding of *B. coli*, as an index of fecal contamination in water.

It is the object of this paper to present a record—incomplete, it may be—of the repeated isolation of organisms simulating these "intestinal forms" in habitats other than those named, and to show that they are actually identical in character with *B. coli* and *Strept. pyogenes*, are abundant, and of constant occurrence on the surface of grains, and in products of milling.

HISTORICAL ACCOUNT.

The opinion that *B. coli* is characteristic of pollution from human sources only was long since proved to be erroneous, as it was shown by Dyar and Keith,[1] Smith,[2] Flint,[3] Belitzer,[4] and Moore and

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Wright[5] to be present in the intestine of many groups of animals. More recently a number of investigators in Europe and America have reported that bacilli in all respects like the colon bacillus from the human intestine, are found in nature where there is no evidence of recent or direct fecal contamination. Kruse[6] and Weissenfeld[7] declared that *B. coli* is present in almost all waters, good or bad.

In 1901 one of us[8,9] isolated from grains and products of milling a considerable number of organisms having all the characteristics of *B. coli*, and Papasotiriu[10] obtained the same results in Europe in an investigation of similar scope. Other workers have also published results which lead to the conclusion that organisms presenting the characteristics of *B. coli* are by no means confined to the intestinal tract of animals, but are widely distributed in nature.

Investigations on the occurrence of *B. coli* on plants, either healthy or diseased, have been made by Gordan,[11] Laurent,[12] and Klein and Houston.[13] The last-named workers examined grains and many food substances for *B. coli*, and although a large number of samples were studied, negative results were obtained in most cases, and it was concluded that *B. coli* was not present on the grains or in the products of milling. All these cultures were isolated after a prolonged preliminary cultivation, generally three days or more, in phenol broth or phenol gelatin—a treatment which we have found generally causes the colon bacillus to be killed out to a large extent, if continued for more than eight hours, and which therefore may explain in part their negative results.

In order to make more certain that the organisms found on grain were not merely bacteria having some of the more striking characteristics of *B. coli*, we undertook in 1902 a careful comparison of cultures derived from the human intestine and from grains. The results of this investigation are embodied in this paper.

In the autumn and winter of 1904 one of us (E. G. S.)[14] studied the organisms occurring on the heads of grain left standing in fields far removed from the sources of pollution with fecal matter, and a little later Metcalf[15] conducted an investigation of the flowers and grains from rice-fields in South Carolina, but where evidences of contamination were not entirely absent.

The investigation of the occurrence of streptococci was begun

in 1894, when the organisms were first reported by Laws and Andrewes.[16] Their sanitary importance was not emphasized until 1899 and 1900, when Houston[17] laid special stress upon the fact that streptococci and staphylococci seem to be characteristic of sewage and animal waste. Horrocks[18] found them in great abundance in sewage and in polluted waters, and showed by experiment that they outlived the colon bacilli in samples of sewage, a subject later explained[19] in 1902, and further discussed by S. K. Baker and one of the writers[20] the following year. LeGros[21] in a monogroph published in 1902 described many streptococci, all derived from the body or from sewage.

The first investigators in this country to call attention to these streptococci were Winslow and Miss Hunnewell[22] in 1901. During the same year they were reported by Gage[23] in the sewage of Lawrence.

The evidence hitherto presented seems to show the streptococci to be associated with animal bodies, occurring either on the surface or within the intestinal tract. The work here described tends to show that these organisms are found in abundance, as is the colon bacillus, on certain substances, at least, outside the body. That they have not been earlier reported is possibly due to a confusion of these organisms and certain bacterium forms, as is suggested by recent work by Heinemann,[27] but more probably because no systematic search for them has been made.

The methods of isolation and investigation, and the comparison of the characteristics of the organisms derived from grain with those of the same species from intestinal sources, may now be most conveniently considered separately.

COMPARISON OF COLON BACILLI FROM THE DIFFERENT SOURCES.

Before making a detailed comparison of the organisms from the two sources, it is necessary to define the characteristics which we have regarded as typical of *B. coli*. These may be expressed as follows:

Form.—Bacillus, 2–3 μ long by 0.5 μ wide, with rounded ends.
Grouping.—Occurs singly, or in short chains or masses.
Motility.—Actively motile; cilia present.
Spore formation.—None.
Staining reactions.—Stains with usual reagents and by Gram's method.

Gelatin plate.—Thin, small, irregular colonies—much as on agar.

Gelatin stick.—Nail growth; small, somewhat spreading colony at surface; gelatin not liquefied.

Agar plate.—Surface colonies opalescent, nearly circular, edges smooth. Submerged colonies clear cut, lenticular. After two or three days surface colonies take irregular grape-leaf forms.

Agar slope.—Twenty hours: luxuriant, moist, opalescent, translucent, white growth narrowing from top to bottom.

Nutrient broth.—Twelve hours: distinct turbidity; 18 hours: slight sediment; after two days, no scum on surface.

Litmus milk.—Litmus reddened and then decolorized in from 12 to 18 hours; milk coagulated; casein not redissolved.

Potato.—Luxuriant, dirty-yellowish growth, later becoming slightly slimy; potato not discolored.

Dextrose broth.—Dextrose fermented in from 6 to 12 hours with formation of acid and gas. Gas ratio $\frac{H}{CO_2} = \frac{2}{1}$, but may vary even with the same stock.

Saccharose broth.—Fermented, with formation of acid and generally some gas.

Lactose broth.—Fermented; acid and gas formed; with less gas, but gas ratio more constant than with dextrose broth.

Maltose broth.—Fermented, with formation of acid and gas; similar to lactose.

Litmus lactose agar plate.—Litmus reddened in less than 24 hours; bubbles of gas formed.

Dunham's solution.—Growth, and pronounced nitroso-indol reaction in three days.

Anaërobic agar streak.—Thin, transparent growth, somewhat less abundant than in aërobic streak.

Lactose neutral red broth.—Gas formed and color generally reduced to canary-yellow (not constant).

METHODS OF ISOLATION USED.

In the earlier experiments cultures of the bacteria examined were obtained by making infusions of the grains in sterile water and plating at once on litmus lactose agar without any preliminary cultivation. Variable numbers of colonies were obtained, some of which were of acid-producing bacteria. These red colonies were then "fished," the masses of bacteria shaken up in sterile water and replated on litmus lactose agar. From the typical isolated colonies on this plate broth cultures were made, and after development these were used as stock cultures for the inoculation of other media—viz., gelatin stab, litmus milk, agar stroke, dextrose broth, nitrate, and Dunham solution. Hanging-drop preparations and stained preparations were made from each culture, and by a comparison of the reactions and morphological features with those of *B. coli*

(intestinal) it was found that 25 out of a total of 47 cultures isolated gave typical colon characteristics, while several more failed in but a single test. Nine cultures were rejected.

The organisms thus obtained, together with a number of cultures of supposed *B. acidi lactici* from various laboratories, were then subjected to actual comparison, side by side with 22 cultures of *B. coli* isolated directly from feces, or from waters known to be sewage-polluted. The fecal bacteria were obtained by preliminary cultivation of the fecal matter in dextrose broth for four to eight hours, and then plating in great dilution on litmus lactose agar. It was found that, if this procedure was used, a nearly pure culture of *B. coli* resulted; but if the preliminary cultivation was prolonged, the colon bacilli were likely to be overgrown by streptococci, and frequently were entirely lost.

In the first series, cultures from the sources mentioned below conform to the following characteristics: Motile, non-spore-forming bacilli producing turbidity in nutrient broth, characteristic growth on gelatin plate, surface and needle-growth in gelatin stab; growth on potato and in closed arm of the fermentation tube; grow at body temperature and are facultative anaërobes; do not liquefy gelatin, casein, or blood serum; produce gas in dextrose, lactose, and saccharose broth; nitrate is reduced, indol formed; milk becomes acid and curdles.

Five cultures of *B. acidi lactici* from the University of Chicago.
Four " from cornmeal.
Six " " buckwheat.
Five " " barley.
Three " " bran.
One culture from flour.
One " " breakfast food.

These cultures give identical results and can in no way be differentiated from the 18 cultures mentioned below, which are of undoubted intestinal origin.

Ten cultures from feces.
Two " " the Mystic River.
One " " " Neponset River.
One " " injected peritoneum.
One " " pool under snow.
One " " driven well in brewery.
One " " polluted brook.
One " " meadow at Framingham.

The following four cultures are without doubt "colon forms," although they fail to give typical reactions in a few cases:

Two cultures from feces failed to reduce nitrates.
One culture from the North River, Salem, did not ferment lactose.
One culture from feces failed to ferment any of the three sugars and rendered milk alkaline.

Thirteen cultures from cornmeal, corn, milk, flour, malt, buckwheat, oats, and laboratory stocks of *B. acidi lactici* were acid-producing organisms that departed widely in character from those just described. These on examination proved to be mostly streptococci, although during this portion of the work but little study was given to them.

FERMENTING POWER.

The fermenting power, as measured by acid production, was taken as a further means of comparing these apparently identical organisms. For this purpose the cultures were grown in 2 per cent dextrose broth at 37° for 48 hours, and the amount of acid determined by titrating 5 c.c. of this solution against $\frac{N}{20}$ NaOH. For fairer comparison the same number of cultures from each of the two sources were taken. Twenty-one cultures of *B. coli* from unpolluted sources required an average of 1.15 c.c. of $\frac{N}{20}$ NaOH to neutralize 5 c.c. of the cultures. Twenty cultures of *B. coli* from feces required an average of 1.13 c.c. of $\frac{N}{20}$ NaOH. Ten cultures of streptococci required an average of 1.35 c.c. of $\frac{N}{20}$ NaOH.

PATHOGENIC PROPERTIES.

As a final test for this series the pathogenic power of the bacteria toward guinea-pigs was studied. Three cultures were chosen at random from each of the two lots, and fresh broth cultures prepared for use as an inoculating medium.

For the first experiment 0.5 c.c. of a culture from cornmeal was injected subcutaneously into a healthy guinea-pig, and a like amount of a culture from an infected peritoneum into another animal of equal weight. The animals exhibited symptoms of fever after about 48 hours, but on the third day appeared more nearly normal.

On being autopsied, each showed a mass of inflamed tissue at the point of inoculation, and from this inflamed portion the bacilli were recovered in large numbers.

As a second test two guinea-pigs were inoculated subcutaneously with 1 c.c. of the same cultures as were used in the previous experiment. In this experiment the animals appeared dull, and their temperature fell slightly in six hours, while at the end of 24 hours the temperature had risen above normal, and the animals were decidedly sick and weak, and showed swelling at the point of inoculation. At the end of 48 hours the temperature had still further risen, but no further change was noticeable. One of these animals was autopsied, the other was kept, and ultimately fully recovered.

A third pair of animals was inoculated intraperitoneally with 1 c.c. of cultures of *B. acidi lactici* and a culture from feces respectively. In this case the animals showed much stronger lesions, although death was not caused in 48 hours. As in the previously described experiments, no difference could be observed in the behavior of the cultures from the two sources. On chloroforming the animals, cultures taken from peritoneum and heart's blood showed the presence of pure cultures of the germs used for inoculation.

Two more animals were infected intraperitoneally, one with another culture of *B. acidi lactici*, and the other with another culture from feces. In this experiment the amount of the dose was 1.5 c.c. in each case. A sharp temperature drop was observed in six hours, and in 24 hours each of the animals was dead. Cultures made from blood and peritoneum gave a pure growth of *B. coli*.

These experiments show conclusively that the pathogenic power of the organisms derived from grain is fully as great as with the intestinal or more nearly parasitic colon bacilli, and, we believe, offers a strong support to the proof of their actual identity. Some more recent work by Gordan[24] has shown the pathogenic properties for white mice of organisms derived from bran.

In all investigations thus far reported some doubt might be cast on the integrity of the samples, or at least there is a possibility of contamination from handling or manufacture. To eliminate this objection, the following work was carried out during the late autumn and winter of 1904. In November a field of rye was found by one

of us in western Massachusetts, which, owing to the scanty growth, had not been cut. The field is of light soil, on a sandy, level, open plain, and situated well back from a little-traveled country road, and far from human habitation. Inquiry showed that the field had not been fertilized,and that no cattle had ranged through the grain during the year. This stand of grain, therefore, may be taken as a typical open-country growth, free from contaminating influences. From this field heads of grain were picked with sterilized forceps and put into sterilized glass tubes. These heads were incubated separately in broth for 24 hours, and then cultures on litmus lactose agar were prepared, following the usual procedure. On December 10 and January 17 two more lots of grain heads were treated in similar manner. In all, 34 heads from this field of rye were studied, and from six of them organisms were isolated giving the reactions of the colon bacillus on all ordinary media and with neutral red lactose broth. It will be recalled that these heads were taken at random over the whole field after they had stood through the storms of the fall and snows of the early winter. Other heads of rye gave acid-producing organisms, but not those exhibiting the typical colon reactions in all respects.

The occurrence of acid- and gas-producing organisms under these conditions has been known for several years, as it was shown by Underwood and one of us[25] in 1897, and again in 1898, that acid-producing organisms of numerous types are of common occurrence upon the ears of sweet corn beneath the husks, and upon the surfaces of peas in the pod, with practically complete protection from contamination from the external world.

Although in the investigations cited only the resistant types of organisms were thoroughly studied, the more recent work shows the strong probability that organisms of the colon and *Strept. pyogenes* types were likewise present. Further evidence on this point is afforded by some experiments begun by us in Wisconsin, in which we examined a large number of kernels of corn from ears carefully selected in the field because of their isolation from polluting substances and further protection from closely packed husks. Although the experiments were not carried out in the great detail of those previously described, the presumptive tests first employed gave strong evidence of the abundance of these bacteria on the grain.

EXAMINATION OF GRAINS FOR STREPTOCOCCUS PYOGENES.

The investigations hitherto described have aimed to point out the common occurrence of *B. coli* on the Gramineæ.

To give greater interest and value to this work, it was deemed desirable to extend its scope by an inquiry into the character and constancy of occurrence of streptococci.

To put the problem in more concrete form: Are the two classes of organisms, colon bacilli and streptococci, of constant occurrence, and if so, do they present any biological relation to each other similar to that existing in sewage or polluted water?

ISOLATION OF BOTH *B. COLI* AND *STREPTOCOCCUS pyogenes*.

It has been shown by one of us that when *B. coli* and *Strept. pyogenes* are both present in a sample of water or sewage, inoculation from the sample into dextrose broth and incubation at 37° gives a development of *B. coli* in the first few (six to twelve) hours, while at the end of 36 to 48 hours the streptococci are predominant. Applying this procedure to grain, we have found that of a very large number of experiments nearly all have shown the occurrence of both organisms.

Thirteen cultures of the colon bacillus isolated from the following sources proved to be identical:

Seven cultures from wheat.
Two " " buckwheat.
Two " " rye.
One culture from barley.
One " " oats.

The purpose of the colon isolations was to confirm earlier results and determine if both kinds develop true to the type.

Plating from the dextrose broth culture on litmus lactose agar as soon as gas formation was well begun gave a predominance of the colon-type organisms, while after 24 hours the streptococci were present in abundance. We have, therefore, not only constant occurrence of both types, but the same course of development in dextrose broth cultures with grains and with sewage—a fact which may have great practical significance in sanitary work.

Comparison of this last series of cultures with the first series will show the constancy in biochemical reactions and morphological features of the colon bacilli from all sources.

This set of cultures of *B. coli* was further compared with the intestinal organisms by a study of the staining relations, with the result that the organisms were found to react toward dyes in the same manner, with the usual staining methods as well as by Gram's method.

COMPARISON OF *STREPTOCOCCUS PYOGENES* FROM GRAIN AND FROM INTESTINAL SOURCES.

Having shown the constant occurrence of streptococci, their comparison with intestinal organisms of the same genus is of interest.

The form of most significance in sanitary work is *Strept. pyogenes*, the "sewage streptococcus." This organism presents the following characteristics:

Form.—Coccus, 1μ in diameter.

Grouping.—Occurs in short chains, often in pairs.

Motility.—Non-motile.

Spore formation.—None.

Gelatin plate.—Small colonies, similar to those on agar; no liquefaction.

Gelatin stab.—Nail growth, apparently made up of isolated colonies; very slight spreading on surface.

Agar plate.—Colonies small; under low power somewhat irregular in form; edges smooth.

Agar streak.—Faint dotted growth in 24 hours.

Broth.—Faint turbidity and perceptible sediment in 18 hours; on shaking, sediment rises in spiral.

Litmus milk.—Twelve hours: slightly acid; litmus slightly decolorized; 18 hours, strongly acid; 36 hours, coagulated.

Potato.—Invisible or hardly perceptible white growth after three days.

Dextrose broth.—Eighteen hours: strongly acid; no gas; sediment and slight turbidity in both arms.

Saccharose broth.—Eighteen hours: sediment and turbidity, but no evidence of change of sugar.

Lactose broth.—Same as dextrose broth.

Maltose broth.—Same as dextrose broth.

Litmus lactose agar plate.—Twelve hours: litmus reddened; colonies small with pink tint as if colored by litmus.

Dunham's solution.—Apparently no growth; no indol produced.

Anaërobic agar streak.—Dotted growth similar to aërobic, but rather less strong.

The cultures isolated from the grains were compared side by side with a set of streptococci isolated directly from feces. Only those organisms showing typical morphological appearance in stained preparation were used in this comparison. Three cultures of *Strept. pyogenes*, eight from rye, six from oats, three from buckwheat, one from wheat, and eight cultures from feces all proved to be alike in their cul-

tural features as well as morphologically. They likewise exhibit the same staining reactions, colorizing readily with the usual stains and also by Gram's method.

COMPARISON OF ACID PRODUCTION.

The comparison of acid-producing power of the organisms was made as a further test of their identity. For this purpose we made use of 1 per cent lactose broth and 1 per cent maltose broth, both made with reaction 0.0 at the beginning of the experiment.

The acidity was determined by titration against $\frac{N}{20}$ NaOH, using phenolphthalein as an indicator.

The acid in the lactose broth cultures was measured after 96 hours, and in the maltose broth cultures after 48 hours.

Five cultures of streptococci from grains required an average of 2.48 c.c. of $\frac{N}{20}$ NaOH to neutralize 5 c.c. of the cultures in lactose broth.

Five cultures from feces required an average of 2.51 c.c. of $\frac{N}{20}$ NaOH cultures in lactose broth.

Six cultures of streptococci from grains required an average of 1.78 c.c. of $\frac{N}{20}$ NaOH to neutralize 5 c.c. of cultures in maltose broth.

Five cultures from feces required an average of 1.83 c.c. of $\frac{N}{20}$ NaOH cultures in maltose broth.

These results show that there is no essential difference in the acid-producing power of the organisms. Taking the averages—2.48, 2.51, 1.78, and 1.83, respectively—it is obvious that the differences are quite within the experimental error in a determination by this method.

Judged by biochemical or microscopical characters and fermenting powers, it is impossible to distinguish between the organisms from the two sources, and we must regard the species *Strept. pyogenes*, as well as *B. coli*, as having a very wide distribution in nature, and not merely associated with animal organisms. Its occurrence appears to us to be in some way correlated with the presence of carbohydrate food, and it is evident that it can obtain this either in the intes-

tine of man or on the developing flower or seed of a plant, especially one in which sugar storage takes place abundantly as in the grains.

SUMMARY AND CONCLUSIONS.

As a result of the comparative investigations which have just been described, we have succeeded in establishing a specific agreement between the organisms corresponding to the graminal *B. coli* and *Strept. pyogenes* and the intestinal *B. coli* and *Strept. pyogenes* in the following ways: (1) in the cultural reactions; (2) in the morphological and biological characteristics; (3) in the fermentative powers and for the colon-like forms; (4) in the pathogenic properties.

The evidence is so positive and so complete as to lead to the conclusion that the identity of these so-called groups is absolute.

It would seem as if the study of the distribution of these germs had hitherto been neglected in much the same way as was, until recently, the case with the tetanus bacillus as well, with one marked difference. The latter has always been thought to be an inhabitant of garden earth, and out of its normal environment when in the human body. Now it has been found to be always present in the fecal discharges of many ruminants,[26] and we come to the question: Which is the normal environment—the earth, the animal body, or both? The colon bacillus, on the contrary, has always been considered the typical intestinal bacillus, and abnormal elsewhere. Our work has led us to suppose that it is normally present either in or on many vegetable tissues, and we are inclined to believe that investigators who have reported *B. coli* in vegetable tissues have not necessarily found germs of immediate intestinal origin, as generally suggested, but simply were not aware of its wide distribution.

The results obtained in this investigation possess considerable interest from both the theoretical and the practical standpoints. Naturally, question first arises as to their origin. Have these organisms been transported through the air as dust, or carried by insects contaminated with animal excrement, and thus gained access to the grain? Assuming this to be the case, it is difficult to explain the numbers and persistence of these forms on grain far removed from contaminating materials in unfertilized fields. While admit-

ting the possibility of this view, it seems to us unlikely. The other alternative is that these organisms are constantly associated with, and of normal occurrence upon, the grain heads; that they find sufficient sustenance to support life, or even to increase in numbers; in other words, that they show a mild form of association or semi-parasitic relation with the plants on which they develop. It seems unreasonable that organisms exhibiting such marked identity in all details of growth and cultural behavior should be regarded as of different species. More likely these organisms bear much the same relation to the sugary heads that *B. subtilis* does to the stalks of hay, or *Strept. hollandicus* to Pinguicula, or the nitrogen-fixing bacteria to the legumes. With this view, it is easy to see how the organisms could find their way to the animal intestine where the temperature and food conditions for rapid development are ideal. Nor is it surprising, in view of the careful and exhaustive search for bacterial proofs of contamination, that the organisms in the intestine should first be sought, and, because of their abundance, regarded as originating in this habitat. The explanation made possible by regarding these species of bacteria as constantly associated with the starch-producing grains is not only simpler, but in our opinion fits in more readily with all the observed facts. Probably many more such associations of certain species of bacteria with plants which can supply their food requirements will be observed in the future, and here is certainly an interesting field for research.

While thus of interest from the general biological standpoint, it is in their bearing on the questions relating to sanitary bacteriological practice that our observations have the utmost importance. Whatever may be the origin of the individuals whence these organisms have been developed, their presence on the grains suggests sources of the colon bacilli and streptococci in water other than direct sewage pollution. Whether the organisms find their way from grain to natural waters can be determined only by an exhaustive study of the bacteriology of unpolluted streams in a new grain-producing region such as western Canada.

Certainly the presence of small numbers of these organisms must be interpreted with the utmost discretion. As a result of careful study, we are not inclined to believe that our results invali-

date the bacteriological examination of waters, for the eastern states at least, except in the immediate neighborhood of grist-mills, etc.; but they certainly render it imperative that great care should always be used in the interpretation of results. Certainly the old and fast rule concerning the significance of the presence of any "intestinal forms" as *prima facie* evidence of sewage pollution must be most discriminately applied.

Whether our results will involve any change in the criteria now in vogue in judging of the character of a water can only be a matter of conjecture until work on the waters of grain-producing regions has been carefully conducted. It seems obvious that at least inspection of watersheds by the trained bacteriologist is necessary, and that no adverse opinion should be based upon the results of a single bacteriological examination without eliminating this possible source of entrance of bacteria of supposedly suspicious character.

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A STUDY OF THE NUMBERS OF BACTERIA DEVELOPING AT DIFFERENT TEMPERATURES AND OF THE RATIOS BETWEEN SUCH NUMBERS WITH REFERENCE TO THEIR SIGNIFICANCE IN THE INTERPRETATION OF WATER ANALYSIS.*

STEPHEN DEM. GAGE.

IN the judgment of the quality of a water many factors must be taken into consideration by the person making the interpretation. Until within a few years it was the custom to base such an interpretation upon the sanitary survey of the source of the water and upon the results of a few chemical determinations. As the value of sanitary analysis became better known and more analyses were made, various discrepancies were noted between the different factors used in the interpretation; and the correct interpretation became more complicated as the number of chemical determinations was increased. With the inception of bacteriological methods of analysis, it was believed that a determination of the number of bacteria would prove a good criterion to the character of a water. Extended examinations, however, proved otherwise, and the determination of numbers of bacteria became merely an additional factor to be used in the interpretation. More recently determinations of specific-groups of bacteria, such as *B. coli*, the sewage streptococcus, and the *B. sporogenes* groups, have been largely exploited, and have proved of more or less value in indicating the character of the water; but as the distribution of these groups has become better understood, the results of these specific bacterial tests have also been found to require interpretation. As the subject stands today, the sanitary quality of a water is usually determined by a critical study of the data obtained, first, from a sanitary survey of the source of the water; second, from the results of a number of chemical procedures; and, third, from the results of bacteriological tests. The chemical procedures of most value are determinations of the nitrogen as free ammonia, as albuminoid ammonia, as

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nitrates, and as nitrites; of the chlorine and of the oxygen consumed from permanganate; although determinations of odor, color, turbidity, iron, hardness, etc., may enter into the consideration of the use of a water for one purpose or another. In the bacterial analysis we have the determination of the numbers of bacteria, usually determination of the presence or absence of bacteria of the colon type, and occasionally determinations of bacteria of the sporogenes or sewage streptococcus type. The analyst having in his possession the above data must know the significance of high or low values of each indicating factor. Even with the number of factors at hand, many of the data are often contradictory, and a correct interpretation of the quality of the water is not always possible.

The weakness in a sanitary survey is that, while it may show pollution, it does not always show whether that pollution is serious, or whether the water has become purified; and, furthermore, it may not reveal sources of pollution which can often be detected by analytical means. The chemical examination can show us, when we know the character of the source of the water, whether the water has or has not been polluted, and the extent of the pollution. It cannot, however, always reveal whether the water in its present state is safe or dangerous for domestic use; and this remains for the bacteriologist to determine. The sanitary survey and the chemical analysis of water have been sufficiently studied, so that our knowledge of the causes of variations and fluctuations in the various factors is well grounded. From the side of the bacterial examination, however, much remains to be investigated. We have a good working knowledge of the significance and fluctuation in the numbers of bacteria in different waters when determined by gelatin or agar plates after incubation at a temperature of 20° C. We are rapidly acquiring a good working knowledge of the true significance of the appearance of *B. coli*, the sewage streptococcus, and *B. sporogenes* in water of various classes. Many bacteriological factors, however, which may be of value have received little or no attention.

It is the purpose of the writer in the present paper to introduce data bearing on the numbers of bacteria which develop on plates incubated at different temperatures, and the ratios between such numbers, for different classes of waters, in an endeavor to show that factors so

obtained may have an important place in judging the character of a water by analytical means. In a large number of laboratories, where the routine work consists in the control of the water supplies of large cities or in the control of water filters, almost complete reliance is placed on the results of bacteriological examinations in judging whether such waters are of the required quality. In such cases any additional factors which will assist in a more accurate judgment, or any change in the procedure which will enable such a judgment to be arrived at more quickly than under present conditions, would be of inestimable value in protecting the consumers from the effects of any sudden change in the quality of such waters.

Media for bacterial counts.—It is well known that gelatin and agar do not show us the total bacterial content of a water, much higher numbers being obtained when other and differently constituted media are employed; and, furthermore, slight changes in the composition of the culture media may cause considerable variations in the numbers of bacteria developing on those media. These points have been very thoroughly investigated by Fuller,[1] Hesse and Nieder,[2] Whipple,[3] G. Hesse,[4] the writer,[5] and others, and their consideration need not be entered upon at this time. Gelatin and agar continue to be the media most commonly employed in quantitative bacterial determinations, for several reasons, the principal ones being that the interpretation obtained by their use is well grounded, and that with the newer media the time required to make a bacterial examination is lengthened instead of shortened. It is largely true, moreover, that while gelatin and agar do not show us the total bacterial content of a water, we are able by their use to obtain a knowledge of a fairly representative section of that bacterial content. The practice as to the use of gelatin or agar varies widely in different laboratories. The principal advantage obtained in the use of gelatin instead of agar is that a determination of the number of liquefying bacteria is possible—a factor of little practical value, except when dealing with sewage and the effluents from sewage disposal works, and which is more than offset by the greater ease with which agar plates may be manipulated.

During more than 15 years it has been the custom to employ agar in routine work at Lawrence, the Lawrence agar being identical in composition with that recommended by the Committee on Standard

Methods of Water Analysis,[6] except that it contains only 1 per cent of agar, instead of 1.5 per cent as recommended by that committee. Litmus-lactose agar as a substitute for gelatin or agar in determining the numbers of bacteria is slowly gaining a foothold in many laboratories, its advantage being that it permits a distinction to be made between the types of bacteria which do and do not produce acid fermentation of lactose, the determinations of the presence and numbers of the fermenting organisms being of considerable significance, as will be shown further on.

Temperature of incubation.—The determination of numbers of bacteria on plates which have been incubated at 20° C. or at room temperature is the usual practice. In many laboratories, where gelatin is employed as the routine medium, the plates are incubated at a somewhat lower temperature, usually about 15° C., in order to prevent the rapid growth of the liquefying bacteria and to minimize the errors due to such liquefaction. In other laboratories, where agar is employed, it is the custom to incubate the plates at temperatures of 23° to 26° C., in the endeavor to obtain higher counts with shorter periods of incubation. The recommendation of the Committee on Standard Methods, that a uniform temperature of 20° C. be employed, has become quite generally adopted in American practice. It is, of course, unnecessary to enter into a consideration of the significance of the numbers of bacteria determined by this procedure at this time.

The value of a determination of the numbers of bacteria which are able to develop at body temperature was first advanced in 1892 by Wurtz.[7] In 1893 Matthews[8] quite clearly demonstrated the distinction in the bacterial content of different classes of polluted and non-polluted waters by the use of lactose agar plates incubated at body temperature, thus confirming the deductions of Wurtz. Routine determinations of the number of bacteria producing acid fermentation of lactose (colon type) on plates incubated at 38° to 40° C. have been made on certain classes of waters since 1896 at Lawrence, and a similar procedure has been adopted by a large number of water bacteriologists. The value of counts of the total number of bacteria developing at 38° to 40° C., however, appears to have been lost sight of in the rush of bacteriologists to study the acid-fermenting types, until Winslow and Niebecker[9] in 1903, after extensive investigations,

arrived at exactly the same conclusions as had Matthews ten years earlier. The peculiar advantage which such determinations have, is that results may be obtained in 18 to 24 hours, or one to three days earlier than is possible with plates incubated at room temperature.

The significance of the numbers of bacteria developing on plates incubated at temperatures above 40° C. has received very little study, although the fact that such bacteria are more or less common has been known for some time. In 1888 Globig[10] first demonstrated that certain types of bacteria capable of growing at temperatures of 50° C. or higher were common in the soil, although Miquel had shown the existence of such bacteria some five years before. In 1889–91 Miquel[11] found organisms of this class, which he named thermophylic bacteria, to be prevalent in polluted waters, but failed to detect them in spring waters. Macfadyen and Blaxall[12] in 1894 demonstrated the presence of thermophylic bacteria in human feces, and Rabinowitschi[13] in the following year found this type of bacteria to be present in the excrement from the majority of domestic animals. More recently, Houston,[14] 1898, has isolated similar types from London sewage and from Thames water, and has called attention to their significance in determining the extent of pollution of a water. Tests for bacteria of this type included in this paper were made on plates incubated at a uniform temperature of 50° C.

So far as has come to the knowledge of the writer, no comparative study has ever been made of the numbers of bacteria in different classes of waters which will develop on plates incubated at 30° C., although it seemed reasonable to believe that some intermediate temperature between 20° and 40° would prove favorable for the growth of a large number of bacteria during a very short period of incubation.

Time of incubation.—The time allowed to elapse between plating and counting varies widely in different laboratories, depending upon local conditions and the medium employed. Owing to the slow development of bacteria at 20° C., it is impossible to obtain counts in less than 48 hours on plates incubated at that temperature. When gelatin is employed, it is usual to count plates on the second or third day, the value of the determinations being obscured by

liquefaction when longer periods of incubation are followed. It is customary to incubate agar plates three to four days, although in some laboratories even longer periods are allowed to elapse before the plates are counted. The recommendation of the Committee on Standard Methods, that a uniform period of incubation of 48 hours be employed, has been quite generally adopted by users of gelatin, but has found less favor among users of agar. At Lawrence it has always been the custom to allow four days to elapse between planting and counting.

With the use of higher temperatures, and the more rapid growth of bacteria at such temperatures, it becomes possible to adopt a shorter period of incubation. Since it is extremely desirable that the results of bacterial determinations be available at the earliest possible moment, the writer has adopted a uniform time of incubation of 24 hours on all plates grown at temperatures higher than 20° C.; consequently the numbers of bacteria included in the discussion and tables beyond, unless otherwise stated, were obtained on plates incubated four days at 20° C., and 24 hours at 30°, 40°, and 50° C, respectively.

The use of ratios between different bacterial counts.—In comparing the analytical results obtained from different waters, or from different samples of the same water, it is quite usual to express the results of such comparison as the ratio, or per cent, which one is of another. The mathematical expression of the ratios between the results of different determinations is less common, and has hitherto been confined to the chemical side of the analysis, so far as the writer knows, no use ever having been made of the ratios between the results of different bacteriological determinations on the same samples.

In the present study we have the results of bacterial counts on agar and on litmus-lactose agar plates which have been grown at four different temperatures, and upon which the number of bacterial colonies have been counted daily until the maximum number of colonies has developed. In addition to the determined numbers of bacteria on each of the plates, and the numbers of bacteria which are able to cause acid fermentation of lactose, both of which determinations may have a place in the interpretation of the character of a

water, we may compute the ratios between each pair of results on the different samples, and by averaging the ratios so obtained for different classes of samples we may ascertain what value each of the different ratios may have in indicating the quality of a water. As a consideration of all of the experimental data, and of all the possible ratios, would occupy more space than is available, many of the data have been excluded, and, in addition to a consideration of the numbers of bacteria determined on agar after four days' incubation at 20° C., and on lactose agar after 24 hours' incubation at 30°, 40°, and 50° C. respectively, and the numbers of bacteria producing red colonies on lactose agar at the same temperatures, only the following ratios will be presented, these ratios being expressed in every case as the per cent which the smaller value is of the greater.

1. The ratios between the total number of bacteria determined on agar after four days' incubation at 20° C., and the numbers of bacteria determined on lactose agar after 24 hours' incubation at 30°, 40°, and 50° C. respectively.

2. The ratios between the total number of bacteria determined on agar after four days' incubation at 20° C. and the numbers of bacteria producing acid fermentation on lactose agar in 24 hours at 30,° 40,° and 50° C. respectively.

3. The ratios between the numbers of bacteria and the number of acid-producing bacteria determined at each temperature as above.

Sources of the data included.—The data used in the discussion and tables have been obtained in part by recomputation of certain of the routine results obtained during the past eight years at the Lawrence Experiment Station, and in part from a special study of the relative counts of bacteria obtained on plates incubated at different temperatures. In addition to the incubators regularly operated at 20° C. and 40° C., we have one spare incubator, which has been operated part of the time at 30° C. and part of the time at 50° C. In considering the significance of the numbers of bacteria and the numbers of acid-producing bacteria determined at the different temperatures, it is therefore necessary to divide the results into two series. The two experiments covered much the same classes of waters, and had determinations of bacteria and acid-producers at 20° C. and at 40° C. common to both experiments. No

direct comparison, however, was possible between the results obtained at 30° C. and 50° C., and we are forced to make such a comparison indirectly through the counts at 20° C. and 40° C. It should be further borne in mind that, while the series containing the 50° C. counts covered a wide range of samples, only five samples were used from each of the several sources, and these samples were all collected within a limited period of time. For this reason the results of this series, while indicative of the results which might be obtained in practice, should not be taken as conclusive evidence of the value of the 50° counts. On the other hand, the results obtained in the 30° series, while covering a smaller range of waters, were obtained from the examination of over 400 samples collected over a period of many months.

Information bearing on the subject at hand has also been drawn from the routine analyses, as follows: About 80 samples from 15 different wells, over 200 samples of sea water from different locations in Boston Harbor, and nearly 1,000 samples of polluted river water upon which bacterial counts at temperatures of 20° and 40° have been made, the counts at 40° being on litmus-lactose agar, and including both total colonies and red colonies, determinations of numbers of bacteria and *B. coli* in over 300 samples of sewage and effluents from sewage filters, and in about 3,700 samples of Merrimack River water from two sources; from which data we have been able to determine very accurately the average ratio of bacteria to *B. coli* for these classes of samples, and in the river water results to study the various factors which have caused changes in the numbers of bacteria and of *B. coli*, and the ratios between the two.

Classification of waters included in the investigation.— During the various investigations 17 different classes of samples have been examined at one time or another, and it is necessary, for a clear understanding of the tables, that a brief description of these classes of samples should be inserted at this point.

Lawrence Street sewage is a strong domestic sewage collected directly from one of the large sewers in the city of Lawrence.—*Regular sewage* is sewage from the same source as the foregoing, conveyed to the Experiment Station through about a mile of 2.5 inch pipe, and differs from the Lawrence Street sewage mainly in the fact that the grosser particles are very largely broken up by abrasion and by passing through the pump.—*Station sewage* is regular sewage diluted with a small proportion of Mer-

rimack River water.—*Septic sewage:* Under "septic sewage" has been included effluents from three septic tanks, all of which treat regular sewage.—*Strained sewage* is regular sewage from which a portion of the suspended matter has been removed by straining through coal.—*Intermittent sewage filters:* Samples from a number of intermittent sand filters operating at low rates with regular sewage or with station sewage are included in the various tables.—*Trickling filters:* Samples from two different trickling filters operating at high rates with regular sewage are included.—*Contact filters:* Samples from three different contact filters have been used, No. 175 operating with regular sewage, No. 176 with strained sewage, and No. 251 with septic sewage.—*Merrimack River water:* Samples from two sources have been used, the source labeled "*Intake*" including samples of the water as it flows upon the Lawrence city filter. Samples labeled "*Canal*" are the river water as received at the Experiment Station after passing through the North Canal. The distance from the Experiment Station to the head of the canal is about one mile, and that from the head of the canal to the Intake of the City Filter about one mile, no sewage entering the river or canal between these points.—*Applied 216:* This is canal water which has been treated with alum and settled before being applied to Filter No. 216—*Water filters:* "*Filter No. 215*" is a mechanical filter operating at a high rate with the above. "*Filters Nos. 8, 220,*" and the "*City Filter*" are slow sand filters operating at low rates, the city filter receiving Merrimack River water designated "Intake," and the others canal water. "*Filters Nos. 243 and 244*" are secondary filters operating with water which has already been filtered.—*Tap water:* This is filtered water from the Lawrence city filter, after being stored in the distribution reservoir and passing through the city service mains.—*Ponds:* Many samples from two large ponds, both used for water supply, have been examined. The watersheds of both of these ponds are under sanitary control, but both are used more or less for pleasure purposes in the summer, and hence are liable to occasional contamination.—*Driven wells:* The samples were from two series of driven wells less than 50 feet deep used as water supply.—*Shallow wells:* Samples from 15 different wells, the results from both good and polluted wells being averaged together. In tables where results from two different wells are given, "No. 1" is of excellent quality, while "No. 2" is badly polluted.—*Springs:* Samples from two different springs, both of good quality, are included.—*Sea waters* include samples collected from a large number of stations during a sanitary survey of Boston Harbor. Most of these samples were more or less polluted with sewage.

Selective action of different temperatures.—In considering the value of counts of total bacteria and of acid-producing bacteria, in addition to the relative numbers obtained by such counts, we should know the selective action of the different temperatures upon the bacterial content of the various classes of waters. We have learned by these experiments that nearly all of the bacteria capable of forming colonies at 20° C. will manifest themselves in four days, this being also true of the acid-producing bacteria. At 40° C. and 50° C. nearly all the bacteria and acid-producers capable of developing at these temperatures are shown by a count made after

24 hours. At 30° C., however, our 24-hour counts show us only 30 to 50 per cent of the bacteria which would be able to produce colonies if the period of incubation were increased to two or three days. In Table 1 is shown the per cent of samples in which bacteria and acid-producing types occurred in waters from different sources when examined by the various methods. The results of determinations of acid-producing bacteria at 20° divide themselves naturally into groups. All of the samples of sewage and effluents from sewage filters, and over 80 per cent of the polluted waters and effluents from sewage filters, contained bacteria of this type the percentage for shallow wells dropping to 70, for pond waters to 60, and for deep wells to 40. The springs differed from the other relatively pure waters in that all samples contained acid-producing bacteria. At 30° C. the percentage of samples showing growth and acid-producing bacteria is generally indicative of the quality of the water, becoming less as the quality of the water improves. The same rule holds for the determinations made at 40° C., the

TABLE 1.

RELATIVE OCCURRENCE OF BACTERIA AND OF ACID-PRODUCING TYPES ON PLATES INCUBATED AT DIFFERENT TEMPERATURES WITH VARIOUS CLASSES OF WATERS.

	Per Cent of Samples Showing						
	Growth at			Red Colonies at			
	30° C.	40° C.	50° C.	20° C.	30° C.	40° C.	50° C.
Sewage	..	100	93	100	..	100	60
Sewage filters—trickling	..	100	100	100	..	100	40
" " —contact	..	100	80	100	..	100	40
" " —sand	..	95	60	100	..	75	17
Merrimack River	94	100	90	91	91	100	70
Applied 216	100	100	100	94	96	97	80
Filter No. 216	77	90	60	90	77	83	60
Other water filters	61	83	55	83	36	57	50
Ponds:	23	48	10	60	10	20	10
Shallow wells	..	80	20	70	..	50	20
Driven wells	..	0	0	40	..	0	0
Springs	..	30	0	100	..	30	0

distinction between samples known to have been polluted recently, even although they have been subjected to purification, and samples from sources whose chance of pollution is more remote, being especially well marked. The driven-well waters showed entire absence of bacteria growing at this temperature. The distinction between sewage and polluted water before filtration, and the effluents from

sewage and water filters, is shown by the smaller per cent of samples from the filtered sources which contain bacteria and acid-producing organisms capable of developing at 50° C. Bacteria of these types occurred only in a small percentage of the samples from ponds and shallow wells, and were entirely absent from samples from springs and driven wells.

Numbers of bacteria, 30° series.—In Table 2 are shown the average numbers of bacteria and of acid-producers obtained with different waters on plates incubated at 20° C., 30° C., and 40° C. From the figures in this table it is seen that the numbers obtained at the different temperatures agree in general with the character of the water under examination. The counts obtained at 30° were larger than those at 40° in every instance when dealing with polluted water, but there was little difference in these counts when dealing with water of good quality. This fact, however, would be an advantage rather than otherwise, since the use of 30° counts would give us a much sharper distinction between good and bad waters than would counts at 40°, and would allow this distinction to be made in a minimum time, 24 hours, as compared with two to four days required to obtain the 20° counts. A rapid method of determining a portion of the bacterial content of waters is especially desirable when dealing with water filters, such filters being controlled largely on the basis of the per cent of the bacteria in the raw water which they remove and the number of bacteria in the filtered water. From the figures in the table we find the percentage removal of bacteria by the Lawrence City Filter to be 99.5, 99.7, and 96.7, as determined by the counts at 20°, 30°, and 40° respectively, and the removal of acid-forming organisms to be 99.1, 99.4, and 95.2 respectively. The percentage efficiency of Filter No. 220 was 98.1, 96.0, and 93.8 respectively, computed from the total bacteria developing at 20°, 30°, and 40° C., and 98.8, 98.6, and 97.7 computed from the acid-forming bacteria developing at these temperatures. The removal of bacteria by Filter No. 216 was 91.0, 96.1, and 82.9 respectively, and the removal of acid-forming bacteria was 95.0, 94.6, and 82.2 respectively, as shown by the figures at 20°, 30°, and 40° C. In other words the percentage removal of bacteria and of acid-formers, as determined by counts at 20° C. after four days'

incubation and at 30° C. after 24 hours' incubation, was very similar, and although the numbers of bacteria determined at 30° C. were smaller than at 20° C., they were fully as significant. The substitution of the 24-hour count at 30° for the usual 20° count in laboratories connected with water filtration plants would result, therefore, in a reduction of the period required to complete a bacterial analysis, without causing any material change in the theories upon which the interpretation of the results are based. The value of counts at 40° C. made at the same time as counts at 20° or 30° should not be underestimated, since the group of bacteria determined at this temperature undoubtedly represent more closely the pathogenic bacterial content of the water than is the case with counts at lower temperatures. It would be inadvisable however, to employ 40° counts exclusively in water work at the present time, until we have a broader understanding of their complete significance, although such counts are used exclusively in some laboratories in the control of milk supplies. The average results of counts at the various temperatures with eight different waters are shown in the following table:

TABLE 2.

AVERAGE NUMBER OF BACTERIA AND ACID-PRODUCERS DEVELOPING AT 20°, 30°, AND 40° C. WITH DIFFERENT CLASSES OF WATER.

	Bacteria per c. c.			Acid-Producing Bacteria		
	20° C. 4 D.	30° C. 24 Hr.	40° C. 24 Hr.	20° C. 4 D.	30° C. 24 Hr.	40° C. 24 Hr.
Canal	4,100	450	81	940	142	44
Intake	5,000	619	122	660	173	42
Applied 216	2,000	203	46	537	55	28
Filter 216	180	8	7	27	3	5
Filter 220	80	18	5	11	2	1
City filter	23	2	4	6	1	2
Pond No. 1	12	0	1	2	0	0
Pond No. 2	17	1	1	1	0	1

Bacterial ratios, 30° series.—In Table 3 are shown the various bacterial ratios for the determinations at 20°, 30°, and 40° C. Certain distinctions between the different waters are brought out by these ratios which do not appear in the numbers of bacteria. In the first two columns are shown the per cent which the numbers of bacteria determined at 30° and 40° after 24 hours' incubation are of the total bacteria determined at 20° after four days' incuba-

tion. From these figures we see that, with one exception, a much greater percentage of the total bacteria are determined at 30° for polluted waters than for the pure waters; that is to say, the distinction between pure and polluted waters is emphasized by the 30° counts. On the other hand, the counts at 40° materially decrease such a distinction, as is shown by the fact that the ratios are greater for the good waters than for the polluted waters. If we assume, however, that the presence of bacteria capable of rapid growth at 40° is an indication that the water contains disease-producing bacteria, the fact that the proportion of such bacteria is greater in the purer waters than in the waters known to be seriously polluted would signify that our filtered waters were not of such excellent quality as their low bacterial content would indicate, which supposition is well worth further study.

No such lesson is apparent in the ratios between the total bacteria at 20° and the acid-producing bacteria at 30° and 40°, the values for the raw waters and filtered waters being much the same. A sharp distinction is noted between the ponds and the other samples in the 30° values, although this distinction does not appear in the 40° values. The ratios between the bacteria growing at each temperature and the number of acid-formers at that temperature appear to be of little value in distinguishing between the different waters. As will be shown later, the chief use of these ratios seems to be in locating errors and abnormal values in the other ratios and in the counts from which they are computed.

TABLE 3.

BACTERIAL RATIOS FOR DIFFERENT CLASSES OF WATERS, 20°, 30°, AND 40° C. SERIES.

	Ratio between Total Bacteria at 20° and Bacteria Developing at		Ratio between Total Bacteria at 20° and Number of Acid-Producing Bacteria at		Ratio between Number of Bacteria at Each Temperature and Acid-Producing Bacteria at That Temperature		
	30° C.	40° C.	30° C.	40° C.	20° C.	30° C.	40° C.
Canal	12.20	1.97	3.46	1.07	23	32	54
Intake	12.38	2.44	3.46	0.84	13	28	34
Applied 216	10.15	2.30	2.75	1.40	27	27	61
Filter 216	4.45	3.89	1.67	2.78	15	38	71
Filter 220	22.50	6.25	2.50	1.25	14	11	20
City filter	8.70	17.40	4.35	8.70	26	50	50
Pond No. 1	0.00	8.33	0.00	0.00	16	0	0
Pond No. 2	5.90	5.90	0.00	5.90	6	0	100

Numbers of bacteria, 50° series.—The average numbers of bacteria and the numbers of acid-producers determined at 20°, 40°, and 50° on five samples each from 26 different sources are shown in Table 4. In general the numbers of bacteria at 20° and 40° and the numbers of acid-producers determined at those temperatures, are large or small as the water is polluted or non-polluted, confirming the findings previously discussed under the 30° series. The most significant results are those obtained for the two shallow wells. The number of bacteria determined at 20° is higher in Well No. 1 than in Well No. 2. Well No. 2 is in a thickly settled community with vaults and cesspools in close proximity, while Well No. 1, situated in an open field upon the top of a hill, is removed from any chance of pollution. Chemical analyses extending over a period of some years indicate that Well No. 1 is free from pollution, and that Well No. 2 is seriously polluted. The sanitary survey and chemical analyses are confirmed by the bacterial count at 40° and by the numbers of acid-producers developing at 20° and 40°, showing that the large numbers of bacteria determined at 20° in Well No. 1 are of a harmless character.

The numbers of bacteria and the acid-formers determined at 50° C. confirm the results of determinations at 20° and 40°, but the distinction between different classes of waters is more marked than by determinations at the lower temperatures. It is noticeable that the 50° bacteria in the effluents from the Contact Filters Nos. 175 and 176 were higher than in the regular sewage which was applied to those filters, and the numbers in the effluent of Contact Filter No. 251 were larger than in the septic sewage with which it was operated. On the other hand, the numbers in the effluents from the trickling filters were small, and this type of bacteria were either entirely lacking or present in insignificant numbers in the effluents from the sand filters. The distinction between the river water and the filtered waters is not very well marked, but a class distinction between the river water and the filtered river waters, and the ponds, wells, and springs, is indicated by the entire absence of this type of organisms in the latter class of waters. The occurrence of 50° acid-producing bacteria is also significant, this type of organisms being absent from the effluents from three out of four of the

intermittent sewage filters, and practically absent from the fourth, while they were present in greater or less numbers in the sewages and in the effluents from the contact and trickling filters through which the sewage passed more rapidly. The presence of such small numbers of this type of bacteria in the polluted river water, and of similar numbers in the effluents from the primary water filters, cannot be accounted for at the present time.

TABLE No. 4.

AVERAGE NUMBER OF BACTERIA AND ACID-PRODUCERS DEVELOPING AT 20°, 40°, AND 50° C. WITH DIFFERENT CLASSES OF WATERS.

	Bacteria per c.c.			Acid-Producing Bacteria		
	20° C. 4 D.	40° C. 24 Hr.	50° C. 24 Hr.	20° C. 4 D.	40° C. 24 Hr.	50° C. 24 Hr.
Regular sewage	2,090,000	557,500	7,700	1,040,000	346,000	4,400
Station sewage	1,676,000	360,000	29,500	1,032,000	283,000	24,900
Septic sewage	485,000	126,500	410	241,000	90,000	240
Sand Filter No. 1	1,640	1,375	2	2,360	1,195	1
" " " 2	35	4	0	29	2	0
" " " 4	1,300	130	1	345	119	0
" " " 9	670	170	2	1,045	154	0
Trickling Filter No. 135	15,500	1,730	154	15,200	1,360	100
" " " 136	23,300	2,030	54	16,000	1,180	20
Contact Filter No. 175	146,600	26,100	8,300	112,400	22,700	8,000
" " " 176	389,000	59,300	8,000	292,000	45,000	8,000
" " " 251	306,000	89,600	485	193,000	46,000	200
Canal	16,400	112	5	6,700	87	2
Intake	16,900	207	4	2,500	134	2
Applied 216	2,800	212	2	1,650	66	1
Filter No. 8	32	3	1	6	1	1
Filter No. 216	715	170	2	259	101	1
Filter No. 243	62	1	0	16	0	0
City filter	150	22	1	14	17	1
City water	64	5	1	11	3	1
Pond No. 1	27	1	0	8	1	0
Pond No. 2	71	8	0	30	5	0
Driven wells	41	0	0	0	0	0
Shallow Well No. 1	1,000	2	0	3	1	0
Shallow Well No. 2	507	72	0	82	55	0
Spring No. 1	40	0	0	6	0	0
Spring No. 2	80	2	0	8	2	0

Bacterial ratios, 50° series.—The bacterial ratios for the different waters included in the 50° series are shown in Table 5. In general the 20°–40° bacteria ratios and the ratios between the 20° bacteria and the 40° acid-producers were much greater for the sewage and the effluents from sewage filters than for the other waters, although there are a few exceptions to this rule. The 20°–40° ratios for the polluted river water in each case were much less than the corresponding ratios for Applied 216 and for the effluents from Water Filters No. 8, 216, and the City Filter, indicating that the removal of the

ordinary water bacteria by coagulation and sedimentation, and by filtration, is greater than is the removal of bacteria capable of developing at 40°, as previously noted in the discussion of the 30° series. The peculiar significance in the ratios between the bacteria and the acid-formers at 20° appears to be in the much larger ratios obtained for sewages and the effluents from sewage filters than for the other waters examined. This distinction does not hold true for the 40° and 50° bacteria-acid-producing-organism ratios, the high and low values being distributed among all classes of waters.

TABLE 5.

BACTERIAL RATIOS FOR DIFFERENT CLASSES OF WATERS, 20°, 40°, AND 50° C. SERIES.

	Ratio between Total Bacteria at 20° and Bacteria Developing at		Ratio between Total Bacteria at 20° and Number of Acid-Producing Bacteria at		Ratio between Number of Bacteria at each Temperature and Acid-Producing Bacteria at That Temperature		
	40° C.	50° C.	40° C.	50° C.	20° C.	40° C.	50° C.
Regular sewage	19.00	0.23	11.60	0.15	48	62	57
Station sewage	21.50	1.80	16.90	1.49	62	79	84
Septic sewage	26.00	0.08	18.50	0.05	50	71	59
Sand Filter No 1	83.80	1.22	72.80	0.61	..	87	50
" " " 2	11.40	0.00	5.70	0.00	83	50	00
" " " 4	10.00	0.08	9.20	0.00	27	92	00
" " " 9	25.30	0.30	23.00	0.00	..	91	00
Trickling Filter No. 135	11.20	0.95	8.80	0.65	98	78	65
" " " 136	8.70	0.23	5.10	0.09	69	58	37
Contact Filter No. 175	17.70	5.70	15.50	5.50	76	87	96
" " " 176	15.20	2.10	11.50	2.10	75	76	100
" " " 251	29.30	0.15	15.00	0.07	63	51	41
Canal	0.68	0.03	0.53	0.01	41	78	40
Intake	1.22	0.02	0.79	0.01	15	65	50
Applied 216	7.56	0.07	2.36	0.04	59	31	50
Filter No. 8	9.40	3.12	3.12	3.12	19	33	100
" " 216	23.80	0.28	14.10	0.14	36	59	50
" " 243	1.61	0.00	0.00	0.00	26	00	00
City filter	14.70	0.67	11.35	0.67	9	77	100
City water	7.80	1.56	4.70	1.56	17	60	100
Pond No. 1	3.70	0.00	3.70	0.00	30	100	00
" " 2	11.25	0.00	7.40	0.00	42	63	00
Driven wells	0.00	0.00	0.00	0.00	00	00	00
Shallow Well No. 1	0.20	0.00	0.10	0.00	00	50	00
" " " 2	14.20	0.00	10.80	0.00	16	76	00
Spring No. 1	0.00	0.00	0.00	0.00	12	00	00
" " 2	2.50	0.00	2.50	0.00	10	100	00

Bacterial determinations at 20° and 40° on polluted waters.—In addition to the results obtained in the 30° and 50° series previously discussed, we have somewhat more extended information regarding the relation between the numbers of bacteria developing at 20° and at 40° C. Throughout 1905 both total colonies and red colonies were counted on all litmus-lactose agar plates. Comparative counts are thus available on some 200 samples of sea waters, and on samples

collected at least three times a week throughout the year from four different polluted sources; these being Merrimack River at the Intake of the City Filter, and, from the North Canal at the Experiment Station, river water which has been treated by coagulation and sedimentation, and river water in which the pollution has been increased by the addition of more sewage. The samples from the Intake and Canal showed very similar results, taken month by month, as is shown in the foregoing tables, and for this reason the Intake samples have been omitted. The samples of sea water exhibit certain peculiarities, and the samples from the three other sources, while similar in character, represent different degrees of pollution, and through them we may gain some insight into the relative fluctuations in the bacteria and in the bacterial ratios.

As the sea waters included samples of varying degrees of pollution, some division of the samples into groups becomes advisable. A number of methods of grouping these samples have been tried, the most satisfactory from the standpoint of the subject-matter of this paper being to place all samples having similar numbers of bacteria in one group, and to average all the results in each group.

The results shown in Table 6 have been obtained in this manner, from which it is seen that the average numbers of bacteria at 20°, the numbers at 40°, and the numbers of *B. coli*—i. e., acid-producers show a similar increase until the numbers of bacteria reach 5,000 per c.c., when the 40° bacteria and the *B. coli* drop to very low numbers and again increase gradually with increasing numbers of bacteria. The ratios between the 20° and 40° bacteria show a corresponding increase until the numbers of bacteria reach 1,000 per c.c., a decrease occurring when the numbers of bacteria are between 1,000 and 5,000, and the ratios becoming extremely small as the numbers of bacteria increase above 5,000. The same peculiarity is noted for the *B. coli* ratios, with the exception that the ratio for an average bacterial content below 100 is greater than the ratio for a bacterial content between 100 and 500. The ratios between the 40° bacteria and the *B. coli* are fairly uniform, fluctuating between 57 and 83. The reasons for the peculiarities above noted cannot be assigned without a careful study of the sources of the various samples and a consideration of all the various factors

influencing the character and quantity of the bacterial content, all of which will be reported elsewhere by another department by whom these samples were collected.

TABLE 6.

NUMBERS OF BACTERIA DETERMINED AT 20° AND 40° C., AND THE CORRESPONDING BACTERIAL RATIOS ON SAMPLES OF SEA WATER.

BACTERIA PER C.C.	20° C.	40° C.		RATIO OF		
	Bacteria per c.c.	Bacteria per c.c.	Acid-Producers	20° Bacteria to 40° Bacteria	20° Bacteria to 40° Acid-Producers	40° Bacteria to Acid-Producers
Less than 100	78	6	5	7.7	6.4	83
Between 100 and 500	370	30	17	8.1	4.6	57
" 500 and 1,000	700	170	110	24.2	15.7	65
" 1,000 and 5,000	2,500	223	155	8.9	6.2	69
" 5,000 and 10,000	5,800	5	4	0.1	0.1	80
" 10,000 and 50,000	19,400	30	19	0.2	0.1	63
Over 50,000	700,000	86	60	...	...	70

The monthly averages of the determinations of bacteria at 20° and 40° C., and the corresponding bacterial ratios on all samples of the canal water, Applied 216 and Applied 219, are shown in Tables 7, 8, and 9. Comparing the canal results with those from Applied 216, the yearly averages show us that treatment of the canal water by coagulation and sedimentation removed about one-half

TABLE 7.

CANAL: AVERAGE MONTHLY NUMBERS OF BACTERIA AT 20° C., AT 40° C., AND OF *B. coli*, AND THE BACTERIAL RATIOS.

1905	20° C. Bacteria per c.c.	40° C.		20°–40° C. Bacteria Ratio	Bacteria 20°–*B. coli* Ratio	Bacteria 40°–*B. coli* Ratio
		Bacteria per c.c.	*B. coli* per c.c.			
January	6.600	114	80	1.73	1.21	70
February	9,800	143	98	1.46	1.00	69
March	5,700	107	63	1.87	1.11	59
April	2,300	46	33	2.00	1.43	72
May	2,600	64	40	2.46	1.54	63
June	8,600	139	81	1.61	0.94	58
July	3,800	151	86	3.96	2.26	57
August	7,100	355	189	5.00	2.66	53
September	14.200	221	155	1.56	1.09	70
October	25,600	647	160	2.53	0.63	25
November	7,900	175	115	2.21	1.45	66
December	6,300	132	97	2.10	1.54	74
Average	8,400	191	100	2.37	1.41	61

of the bacteria contents of the water. On the other hand, the ratios show us that the removal of bacteria capable of growing at 40° and of *B. coli* was less than that of the total bacteria. Furthermore,

while the fluctuation in the numbers of total bacteria, of bacteria growing at 40°, and of *B. coli*, was less in the Applied 216 than in the canal, the fluctuation in the ratios between these numbers was very much greater.

Comparing the canal with Applied 219, we find that by adding a small proportion of sewage to the water we have increased our bacterial content, as shown by higher values on all three determinations;

TABLE 8.

APPLIED 216: AVERAGE MONTHLY NUMBERS OF BACTERIA AT 20° C., AT 40° C., AND OF *B. coli*, AND THE BACTERIAL RATIOS.

1905	20° C. Bacteria per c.c.	40° C.		20°–40° C. Bacteria Ratio	Bacteria 20°–*B. coli* Ratio	Bacteria 40°–*B. coli* Ratio
		Bacteria per c.c.	*B. coli* per c.c.			
January	5,000	69	39	1.28	0.78	47
February	7,900	76	46	0.96	0.59	62
March	2,300	44	24	1.91	1.04	55
April	1,500	25	18	1.67	1.20	72
May	1,600	39	23	2.43	1.43	59
June	5,100	105	66	2.06	1.29	58
July	1,000	58	27	5.80	2.70	47
August	1,400	104	63	7.45	4.50	61
September	4,500	223	162	4.95	3.60	73
October	8,600	149	96	1.74	1.11	64
November	4,800	107	71	2.20	1.48	66
December	3,800	72	43	1.88	1.13	60
Average	4,000	89	57	2.86	1.74	60

but by comparing our ratios we find that we have increased the class of bacteria developing at 40°, in which must be included the disease germs in a much larger proportion than we have increased the total bacterial content. The fluctuation in the numbers of the different classes of bacteria and the fluctuation in the ratios between these

TABLE 9.

APPLIED 219: AVERAGE MONTHLY NUMBERS OF BACTERIA AT 20° C., AT 40° C., AND OF *B. coli*, AND THE BACTERIAL RATIOS.

1905	20° C. Bacteria per c.c.	40° C.		20°–40° Bacteria Ratio	Bacteria 20°–*B. coli* Ratio	Bacteria 40°–*B. coli* Ratio
		Bacteria per c.c.	*B. coli* per c.c.			
May	20,200	507	339	2.47	1.67	67
June	12,800	260	198	2.03	1.54	76
July	17,800	850	828	4.76	4.65	97
August	8,800	537	251	6.10	2.85	48
September	44,700	616	357	1.38	0.80	58
October	46,400	1034	595	2.21	1.28	58
November	28,200	1746	791	6.20	2.80	45
December	26,000	778	531	2.99	2.04	68
Average	25,600	791	486	3.52	2.20	65

numbers correspond fairly well with the probable difference in the character of the two waters. In other words, our ratios indicate that both Applied 216 and Applied 219 were more dangerous from a sanitary standpoint than the difference between their total bacterial counts and the count on the canal water would indicate.

Bacteria–B. coli ratios on Merrimack River water.—We will now consider the ratio between the numbers of bacteria and the numbers of *B. coli*, this being the one of the new factors here introduced upon which we have the most extensive data. Routine determinations of bacteria and *B. coli* have been made on samples of Merrimack River water from the Intake during a period of seven years, and on samples from the canal during a period of eight years. The average monthly numbers of bacteria and *B. coli* in samples from these two sources have been published annually in the reports of the Massachusetts State Board of Health, and it is unnecessary to reproduce them here. The ratios between the bacteria and *B. coli* computed from the monthly averages for these two sources are shown in Tables 10 and 11. The use of the monthly averages tends to eliminate abnormal values and fluctuations, and to give us results which are very

TABLE 10.

AVERAGE MONTHLY BACTERIA–*B. coli* RATIOS ON SAMPLES FROM THE MERRIMACK RIVER AT THE INTAKE OF THE CITY FILTER, 1899–1905, INCLUSIVE.

	1899	1900	1901	1902	1903	1904	1905	Average
January	0.57	0.69	1.64	0.29	0.26	0.61	0.71	0.68
February	0.53	0.47	3.28	0.61	0.33	0.71	0.84	0.97
March	0.30	6.30	0.78	0.44	0.98	0.57	0.95	1.47
April	0.41	1.30	2.43	0.38	1.09	0.44	1.06	1.01
May	1.10	1.56	1.43	0.85	1.41	1.71	1.37	1.35
June	1.57	1.48	0.73	2.40	0.78	1.25	0.91	1.30
July	1.97	1.51	0.61	0.43	1.78	1.56	1.46	1.33
August	0.16	1.31	1.06	1.05	1.65	0.97	1.39	1.08
September	0.83	0.93	0.50	1.07	0.37	0.66	1.40	0.82
October	0.24	0.22	0.53	0.53	0.44	1.66	1.10	0.67
November	0.61	1.86	4.07	1.92	1.32	0.70	1.84	1.76
December	1.43	1.93	0.16	1.03	0.66	0.64	1.06	0.99
Average	0.81	1.63	1.44	0.92	0.92	0.96	1.17	1.12
Maximum	1.97	6.30	4.07	2.40	1.78	1.71	1.84	6.30
Minimum	0.16	0.22	0.16	0.29	0.26	0.44	0.71	0.16

nearly normal, and the fluctuations occurring from month to month must be considered normal fluctuations. These fluctuations from the normal and the effect of various factors in producing normal fluctuations will be considered later.

The numbers of bacteria and of *B. coli* in the Intake samples have usually been somewhat greater than those in the canal samples.

A study of the bacteria-*B. coli* ratios reveals that those ratios also have been constantly higher for the Intake samples than for the canal samples, and that there has been a greater uniformity in the bacterial content of the Merrimack River water after it has passed through the canal and the short distribution pipes to the experimental filters at the Experiment Station, taking the results year by year, and month by month, than was the case with the same water as it was applied to the Lawrence City Filter.

The average ratio on all samples collected from the Intake during the entire seven years was 1.12, the lowest yearly average being 0.81, in 1899, and the highest 1.63, in 1900. The lowest monthly average occurred in August, 1899, and again in December, 1901, when the ratio was 0.16, and the highest monthly average, 6.30, occurred in March, 1900. The least variation in the monthly ratios for any one year occurred in 1904, the difference between the highest and lowest values in that year being 1.27. The greatest variation in the monthly ratios in any year occurred in 1900, when the difference between the highest and lowest ratios was 6.08.

TABLE 11

AVERAGE MONTHLY BACTERIA—*B. coli* RATIOS ON SAMPLES FROM THE MERRIMACK RIVER FROM THE NORTH CANAL, 1898-1905 INCLUSIVE.

	1898	1899	1900	1901	1902	1903	1904	1905	Average
January	0.80	0.83	0.47	2.56	0.52	0.39	0.95	1.21	0.97
February	0.59	0.45	0.60	2.42	0.61	0.44	1.22	1.00	0.92
March	0.45	0.29	0.61	1.41	0.14	0.61	0.37	1.11	0.62
April	1.20	0.65	0.70	1.10	1.86	1.06	0.50	1.43	1.06
May	0.79	0.82	0.98	0.83	1.37	0.95	0.81	1.54	1.01
June	0.56	0.97	1.96	0.44	0.60	0.65	0.93	0.94	0.88
July	1.12	2.50	2.59	0.47	0.43	0.90	2.32	2.26	1.57
August	2.12	0.78	1.08	0.16	1.94	0.87	1.86	2.66	1.43
September	1.75	0.41	0.71	1.32	0.97	0.57	0.88	1.09	0.96
October	0.55	0.11	0.46	0.08	0.91	0.70	1.00	0.63	0.56
November	1.07	0.62	1.24	0.27	0.98	1.40	0.86	1.45	0.99
December	0.65	0.93	1.53	0.31	0.66	0.49	0.38	1.54	0.81
Average	0.97	0.80	1.08	0.95	0.92	0.75	1.01	1.41	0.98
Maximum	2.12	2.50	2.59	2.56	1.94	1.40	2.32	2.66	2.66
Minimum	0.45	0.11	0.46	0.08	0.14	0.39	0.37	0.63	0.08

The average ratio on all samples from the canal during the entire period of eight years was 0.98, the lowest yearly average being 0.80, in 1899, and the highest 1.41, in 1905. The lowest monthly ratio occurred in October, 1901, when the ratio was 0.08, and the highest occurred in August, 1905, when the ratio was 2.66. The least variation in the monthly ratios during any one year occurred in

1903, the difference between the highest and lowest values being 1.01, and the greatest variation in the monthly ratios occurred in 1901, the difference between the highest and lowest values being 2.48.

Normal ratios.—That the labor of computing the ratios between the various bacterial constituents is repaid by the additional information acquired as to the character of the water, is clearly demonstrated in the preceding chapters. The bacterial ratios may also be made to serve as a check upon the bacterial determination, and by pointing out fluctuations in the bacterial contents of different samples of water from the same sources, which would otherwise have passed unnoticed, indicate the direction in which further investigations must be made to convert the hitherto unexplainable discrepancies between analytical results into significant facts. In order that we may have a clear understanding of the significance of the different ratios, and of the relative numbers from which they are computed, it is important that we should know what are the normal ratios for different classes of waters. The determination of the normal value for any set of variable characters is distinct from the determination of the average of those characters, and consists in arranging the individual values in groups, each group containing all values which are between certain limits, and the normal value for characters which have been arranged in this manner will then lie within the limits of the group which contains the greatest number of individual values.

In Tables 12–15, inclusive, certain of the bacterial ratios have been grouped in this manner, the size of each group being expressed as the percentage which the number of samples included in the group is of the total number of samples examined. The various 50° ratios have been omitted, for the reason that a fair normal value cannot be obtained by the group method when the investigation covers less than 25 samples. On the other hand, determinations of bacteria and *B. coli* in Merrimack River water have been made on thousands of samples, while on certain other classes of samples many hundred determinations have been made, with the result that the normal ratios for such waters are correspondingly accurate.

The normal ratio between the 20° and 30° bacteria for the Merrimack River samples appears to be about 5, 40 per cent of the samples having ratios between 1 and 5, 9 per cent being below 1, and the remaining 51 per cent being well distributed above 5. As the waters become purer, the ratios become more varied. The normal limit for Applied 216 is still between 1 and 10, but is apparently somewhat higher than in the case of the river water. The normal limit for the effluent from Filter No. 216, like the river water, is between 1 and 5, but the majority of the other ratios fall below rather than above those limits. The purer effluents from the water filters operating at low rates are characterized by the large percentage of samples having ratios of 0, and by the distribution of the remaining samples among the higher ratios, which fact is still further emphasized when we come to the pond waters.

The normal ratio between the 20° and 40° bacteria again falls between 1 and 5 for samples from the Merrimack River and from Applied 216. In the same group must be included the effluent from mechanical Filter No. 216. The ratios for the other water filters are more widely distributed, 19 per cent of the samples not showing any growth at 40°, and 37 per cent having ratios above 15. The group

TABLE 12.

DISTRIBUTION OF THE RATIOS BETWEEN 20° BACTERIA AND BACTERIA AT 30° AND 40° AMONG DIFFERENT SAMPLES FOR VARIOUS CLASSES OF WATERS.

	Per Cent of Samples Having Ratios					
	Of 0	Less than 1	Between 1 and 5	Between 5 and 10	Between 10 and 15	Above 15
FOR RATIOS BETWEEN 20° AND 30° BACTERIA.						
Merrimack River	2	7	40	15	13	23
Applied 216	0	19	31	19	12	19
Filter No. 216	24	8	40	16	8	4
Other water filters	41	0	9	19	9	22
Ponds	77	0	3	5	5	10
FOR RATIOS BETWEEN 20° AND 40° BACTERIA.						
Merrimack River	0	15	63	17	0	5
Applied 216	0	15	54	19	12	0
Filter No. 216	12	0	56	24	4	4
Other water filters	19	0	3	19	22	37
Sea waters	19	9	20	23	23	6
Ponds	49	0	3	15	10	23
Wells	42	18	20	9	4	7

of sea waters, containing, as it does, samples probably not exposed to pollution, and samples which are grossly polluted, shows a more uniform distribution of ratios than any of the other classes of waters, although the normal ratio for samples containing bacteria which are able to grow at 40° appears to lie between 5 and 10. The distinction between the pond waters and the well waters is quite marked Forty-nine per cent of the pond samples and 42 per cent of the well samples did not contain any bacteria growing at 40° C. Of the samples which did contain bacteria of this type, nearly all of the pond samples have high ratios, while the ratios for the well samples are usually very low. The percentage distribution of the different ratios between the 20° bacteria and the bacteria determined at 30° and 40° for different classes of waters is shown in Table 12.

The normal ratios between the 20° bacteria and the 30° acid-producing organisms follow much the same rule as the 20°–30° bacteria ratios, the distinction between the different classes of water, however, being much more sharply marked. The normal ratio for Merrimack River water lies between 1 and 5, and with a large majority of the ratios above 5, falling below 10. With the Applied 216, while the normal ratio lies between the same limits as for the river water, the other ratios are about evenly distributed above and below those limits. Again, with the effluent from Filter No. 216 the normal ratio is between 5 and 10, but the number of samples having ratios

TABLE 13.

DISTRIBUTION OF RATIOS BETWEEN 20° BACTERIA AND 30° ACID-PRODUCING BACTERIA AMONG DIFFERENT SAMPLES FOR VARIOUS CLASSES OF WATERS.

	Per Cent of Samples Having Ratios					
	Of 0	Less than 1	Between 1 and 5	Between 5 and 10	Between 10 and 15	Above 15
Merrimack River	0	10	50	29	8	3
Applied 216	8	23	42	23	4	0
Filter No. 216	36	12	44	4	4	0
Other water filters	66	0	6	13	6	9
Ponds	90	0	3	7	0	0

above 5 is small, and a considerable percentage of the samples did not show acid-producing bacteria. As we pass to the effluents from the slow water filters, and thence to the pond waters, the percentage of samples free from acid-producing bacteria increases, while the normal

ratios for such samples as do contain bacteria of this type are above 5. The figures are shown in Table 13.

Normal bacteria–B. coli ratios.—In determining the normal ratio between the 20° bacteria and the *B. coli*, our available data cover a wider variety of sources and a larger number of samples than is the case of the ratios previously discussed. With raw sewages 57 per cent of the samples examined have ratios between 1 and 5, and 26 per cent of the samples have ratios between 5 and 10, the other ratios being distributed on both sides of these limits. The normal ratio for raw sewages, then, is probably just below 5. The treatment of sewage by septic action or by straining tends to eliminate abnormal ratios, as is shown by the fact that 75 per cent of the septic sewage samples and 83 per cent of the strained sewage samples have ratios between the 1 and 5. A somewhat smaller percentage of the ratios for the effluents from sewage filters is found between 1 and 5, although the normal ratios for these classes of samples still remain within those limits. A distinction between the effluents of the sand and trickling filters, whose action is entirely aërobic, and the contact filters, whose action is partly anaërobic, is manifest by the considerable percentage of samples of the former classes which have ratios less than 1, as compared with the small percentage of the latter class. The distribution of the ratios over a broader scale is also notable, indicating the fluctuating character of the types of bacteria contained in the effluents from all types of sewage filters. The normal ratios for the Merrimack River water and Applied 216 are also between 1 and 5, with a large majority of the ratios outside these limits, falling below 1. The effluent from Filter No. 216 has a normal ratio between 1 and 5, with 20 per cent of the samples having a ratio of 0, while the effluents from the slow water filters are characterized by the fact that 50 per cent of the samples have a ratio of 0, the other ratios being fairly well distributed, with a normal ratio somewhat above 5. The variable character of the sea-water samples is evident from the distribution of the ratios. With this class of samples the normal ratio again falls between 1 and 5, 39 per cent of the samples having ratios between those limits, with 21 per cent lying between 5 and 10, 14 per cent falling below 1, and 19 per cent with a ratio of 0. The difference

between the pond and well waters and the other classes of samples is indicated by the large number of samples which do not contain acid-producing bacteria at 40° C. A distinction occurs between the pond and well waters, as shown by the fact that the ratios for the former are distributed among the higher values, while the ratios for the latter are distributed among the lower values. The normal ratio is not apparent for the pond waters, but the normal ratio for such samples of well water as contained bacteria of this type falls between the limits previously noted for other classes of samples; that is, between 1 and 5. The distribution of the bacteria–*B. coli* ratios for all samples from these 13 different sources is shown in Table 14:

TABLE 14.

DISTRIBUTION OF BACTERIA–*B. coli* RATIOS AMONG DIFFERENT SAMPLES FOR VARIOUS CLASSES OF WATERS.

	Per Cent of Samples Having Ratios					
	Of 0	Less than 1	Between 1 and 5	Between 5 and 10	Between 10 and 15	Above 15
Lawrence sewage	0	6	57	26	8	3
Septic sewage	0	10	75	10	5	0
Strained sewage	0	17	83	0	0	0
Sand filter effluents	0	31	41	11	5	12
Contact " "	0	5	48	22	13	12
Trickling filter effluents	0	26	44	11	11	8
Merrimack River	0	36	60	2	2	0
Applied 216	4	27	58	11	0	0
Filter No. 216	20	0	68	8	4	0
Other water filters	50	3	13	19	6	9
Sea waters	19	14	39	21	2	5
Pond waters	82	0	3	5	3	7
Wells	82	5	12	1	0	0

In Table 15 is shown the distribution of the ratios between the bacteria and the acid-producers developing at 20°, 30°, and 40° C. In computing the values in this table only samples are included which contained bacteria capable of development at the given temperature. The difference between these ratios and the ones previously discussed consist in their much wider distribution, and this has made necessary the grouping of the ratios between somewhat wider limits.

The normal ratios between the bacteria and acid-producers at 20° for Merrimack River water and Applied 216 appear to be about 20 in each case. The normal ratio for the effluent from Filter No.

216 is well below 20, probably about 10; while the ratio for other filtered waters appears to be somewhat higher, lying between 15 and 20. The pond waters are characterized by very low ratios, the normal ratio being about 1, 41 per cent of the samples having ratios below 1, and 31 per cent between 1 and 20.

The ratios between the bacteria and acid-producers at 30° are higher than the corresponding ratios at 20°. The normal ratios for the river water and Applied 216 lie between 40 and 80, probably being about 60 in each case. A majority of the samples from Filter No. 216 had ratios above 80, with 32 per cent of the samples having ratios between 20 and 40, the remainder of the samples being divided in two equal groups, having ratios between 1 and 20, and between 40 and 60. With the effluents from the slow sand filters we again find a majority of the samples having ratios above 80, with 36 per cent of the samples evenly divided into two groups, with ratios between 20 and 40, and between 40 and 60, respectively. Seventy-five per cent of the pond-water samples have ratios above 80, while the remaining 25 per cent have ratios between 40 and 60. A distinction between the different classes of water is seen in the number of samples having ratios above 80, about 25 per cent of the polluted water samples, 50 per cent of the filtered waters, and 75 per cent of the pond waters being so characterized.

Normal ratios between 40° bacteria and acid-producers.—In studying the ratios between the bacteria and acid-producers at 40° we have available a large variety of sources and more samples from each source, so that our results are more conclusive. The normal ratio for the river water appears to lie just above 60, while that for Applied 216 appears to be between 70 and 80. Over 55 per cent of the Filter No. 216 samples have ratios above 80, thus placing the normal ratio for this water just above 80. With the effluents from the slow water filters we find the ratios more uniformly distributed, and although the normal ratio appears to be about 60, its location is not distinctively marked. The pond water normal ratio is located between 70 and 80, and the ratios are distributed less widely all being above 20, as was the case with the samples from Applied 216 and Filter No. 216. The sea waters are again divided into two groups, 19 per cent of the samples having ratios less than 1, while

nearly all the remaining samples had ratios above 40. The normal ratio for this latter group of sea-water samples is probably between 50 and 60. The well-water samples are characterized by the fact that 68 per cent of the samples have ratios less than 1, the ratios for the remaining samples being distributed in a fairly uniform manner.

TABLE 15.

DISTRIBUTION OF THE RATIOS BETWEEN THE TOTAL BACTERIA AND THE ACID-PRODUCING BACTERIA AT 20°, 30°, AND 40° AMONG DIFFERENT SAMPLES FOR VARIOUS CLASSES OF WATERS.

	PER CENT OF SAMPLES HAVING RATIOS					
	Less than 1	Between 1 and 20	Between 20 and 40	Between 40 and 60	Between 60 and 80	Above 80
PLATES INCUBATED AT 20° C.						
Merrimack River	10	38	40	10	0	2
Applied 216	12	31	31	19	7	0
Filter No. 216	12	60	16	4	0	8
Other water filters	19	31	41	0	9	0
Ponds	45	31	13	3	3	5
PLATES INCUBATED AT 30° C.						
Merrimack River	0	8	13	28	28	23
Applied 216	0	5	16	25	29	25
Filter No. 216	0	6	32	6	0	56
Other water filters	0	9	18	18	0	55
Ponds	0	0	0	25	0	75
PLATES INCUBATED AT 40° C.						
Merrimack River	0	5	13	23	42	17
Applied 216	0	0	16	20	32	32
Filter No. 216	0	0	10	10	25	55
Other water filters	0	12	13	25	31	19
Ponds	0	0	14	14	29	43
Sea waters	19	1	2	48	18	12
Wells	68	2	4	11	4	11

Causes of variation in bacterial contents of Merrimack River water.—As fluctuating factors which are most liable to influence the numbers of bacteria and *B. coli*, and the ratio between bacteria and *B. coli*, in the water from such a source as the Merrimack River, we have the volume of water flowing in the river, the temperature of the water, and the amount of dissolved oxygen in the water. For a clear understanding of the influence of these three factors it is first necessary to consider the influence of the various factors upon one another. It is quite generally understood that the amount of oxygen dissolved in water varies inversely as the temperature, a greater amount of

oxygen being present in the winter, when the water is cold, than in the summer, when the water is warm. The relationship between these two factors is shown quite clearly in Table 16. A study of the temperatures of the water, and of the amounts of dissolved oxygen at times of high and low water, reveals the fact that the average temperature was high, and the average amount of dissolved oxygen was low, when the river was low, these factors decreasing and increasing respectively as the volume of flow in the river increased. The average results of eight years' determinations of the temperature and dissolved oxygen, arranged according to the volume of water flowing in the river, are shown as follows in Table 16:

TABLE 16.

RELATION OF TEMPERATURE AND DISSOLVED OXYGEN TO VOLUME OF MERRIMACK RIVER.

Flow of River—Cubic Feet per Square Mile of Watershed	Temperature of Water	Dissolved Oxygen —Parts per 100,000
Less than 1	60	0.73
Between 1 and 2	48	1.09
" 2 and 4	47	1.11
Above 4	46	1.13

The effect of different amounts of dissolved oxygen in the water is shown quite clearly in Table 17, in which the bacterial results have been arranged according to the amount of oxygen present. The numbers of bacteria and of *B. coli* were both at a maximum when the dissolved oxygen in the water was between 0.50 and 0.75 parts per 100,000. As the amount of oxygen increased or decreased from these limits, the numbers of bacteria and *B. coli* decreased, the numbers of *B. coli* decreasing much more rapidly with the increase

TABLE 17.

RELATION BETWEEN AMOUNT OF DISSOLVED OXYGEN AND THE BACTERIAL CONTENTS OF MERRIMACK RIVER WATER.

Dissolved Oxygen-Parts per 100,000	Bacteria per c.c.	*B. coli* per c.c.	Bacterio-*B. coli* Ratio
Less than 0.50	6,200	74	1.42
Between 0.50 and 0.75	10,000	104	1.16
" 0.75 " 1.00	6,000	47	0.87
" 1.00 " 1.25	4,200	37	0.88
More than 1.25	5,200	30	0.59

in dissolved oxygen than did the bacteria. This is shown by the bacteria-*B. coli* ratios in the last column of the table, the ratios

decreasing in proportion as the amount of oxygen in the water increased.

The seasonal distribution of *B. coli* in samples of Merrimack River water has been discussed in a former publication[15] in which it was shown that the average numbers of *B. coli* were higher during the summer months than during the winter months. A similar variation can be noted in Table 18, in which the bacteria and *B. coli* and the ratio between the two have been arranged according to the temperature of the water at the time the samples were collected. The maximum numbers of both bacteria and *B. coli* occurred in samples from both locations when the temperature of the water was between 60° and 70° F., and the minimum numbers of bacteria were found when the temperature was between 40° and 50° F. The temperatures at which the minimum numbers of *B. coli* were found in samples from the two locations do not agree, being lowest in the canal water when the temperature was between 40° and 50° F., and lowest in the Intake samples when the temperature was between 30° and 40° F. The bacteria–*B. coli* ratios were highest when the temperature was highest and lowest when the temperature was lowest, the values for intermediate temperatures, however, appearing to follow no definite curve.

TABLE 18.

RELATION BETWEEN TEMPERATURE AND BACTERIAL CONTENTS OF MERRIMACK RIVER WATER.

Temperature of Water—Degrees F.	Bacteria per c.c.		*B. coli* per c.c.		Bacteria–*B. coli* Ratio	
	Canal	Intake	Canal	Intake	Canal	Intake
Below 40°	5,800	8,800	43	56	0.83	0.72
Between 40° and 50°	4,000	4,500	36	58	0.91	1.10
" 50° and 60°	6,400	9,500	44	58	0.88	0.99
" 60° and 70°	11,300	15,300	100	110	1.08	0.97
Above 70°	5,200	6,700	70	82	1.39	1.21

The averages of bacterial determinations and the bacteria–*B. coli* ratios, arranged according to the volume of water flowing in the river, are shown in Table 19. Both bacteria and *B. coli* decreased as the volume of the river increased. We should expect this to be the case in a river such as the Merrimack, in which a large proportion of bacteria and *B. coli* are contributed by the sewage entering that river, the effect of dilution overbalancing other factors, such as wash-

ings from cultivated fields, etc., when averages of a large number of samples extending over a considerable period are included. The ratios between the bacteria and *B. coli* on samples from the two sources do not agree, the highest ratios being obtained for the canal samples when the river was low, and the lowest ratios when the river was high. With the Intake samples, however, the lowest ratios occurred at a time of medium high water, and the highest at extreme high water.

TABLE 19.

RELATION BETWEEN VOLUME OF FLOW AND THE BACTERIAL CONTENT OF MERRIMACK RIVER WATER.

Flow of River, Cubic Feet per Second per Square Mile of Watershed	Bacteria per c.c.		*B. coli* per c.c.		Bacteria-*B. coli* Ratio	
	Canal	Intake	Canal	Intake	Canal	Intake
Less than 1	7,500	10,800	66	88	1.07	0.97
Between 1 and 2	6,800	6,200	50	51	0.73	0.99
" 2 and 4	3,600	5,600	29	39	0.83	0.56
Above 4	3,400	3,100	16	29	0.63	1.07

CONCLUSIONS.

The apparent discrepancies which have occurred between the results of bacteriological and of chemical analysis of water have caused a reasonable doubt in the minds of many persons, having occasion to use such results, as to the practical value of the bacteriological determinations. That such discrepancies do exist cannot be denied, and that the bacteriological results instead of the chemical results should be doubted is natural, considering that the complete chemical analysis is composed of a number of individual factors, each of which has received long and careful study, while the bacteriological procedure is confined to a determination of the numbers of bacteria, and of the presence or absence of one specific type of bacteria, i. e., the colon type. If the chemical analysis of water were confined to a determination of the total nitrogen content, instead of dividing that nitrogen content into its constituent parts—free ammonia, albuminoid ammonia, nitrates, and nitrites—and only a qualitative test were made for chlorine, the interpretation of the character of the water from the chemical results would be as frequently in error as when a similar interpretation based on the usual bacteriological results is attempted. If, on the other hand, complete and

varied data regarding the bacterial contents of a water could be obtained, the apparent discrepancies would cease to exist, and the chemical and bacteriological analyses would supplement and confirm one another, rendering the correct interpretation of the quality of the water a comparatively simple matter. The object of the present paper has been to supply a portion of the information necessary for the etablishment of bacteriological procedures by which a more thorough knowledge of the bacterial content of the water may be obtained. Nearly all of the information desired concerning the bacterial content of water may be obtained by the use of selective media, by the use of selective temperatures, or by a proper combination of the two. In the present investigation the selective action of four different temperatures, 20°, 30°, 40°, and 50° C., and two different media, regular agar, and litmus-lactose agar, in determining the bacteriological contents of a number of different kinds of water, have been studied; and while the results obtained have been in many cases inconclusive, and in other cases too few in number to warrant the drawing of any far-reaching conclusions, they indicate in a measure the procedures which must be followed in order to place the bacteriological analysis of water on the same plane as the chemical analysis.

The results of the investigation may be summarized as follows: The numbers of bacteria determinable upon agar or gelatin are very closely approximated by the numbers determined upon litmus-lactose agar, while the substitution of the latter medium for the former allows of the simultaneous determination of the total bacteria and of the acid-producing bacteria without appreciably increasing the labor involved in the determination. The numbers of the two classes of bacteria so determined indicate more completely the character of the water than would the numbers of either class determined alone.

It is, of course, unnecessary to discuss the significance of the numbers of bacteria determined at 20° C. The number of acid-producing organisms determined at 20° C., however, is an important check upon the total numbers. In one case we saw that with two well waters, one polluted and one not polluted, the numbers of bacteria in the pure well water were about twice as great as in the polluted water, but the numbers of acid-producing bacteria showed the high numbers for the pure water to be misleading.

The numbers of bacteria and of acid-producing organisms determined on litmus-lactose agar at 30° C. after 24 hours' incubation are smaller than the numbers determined at 20° C. after two to four days' incubation; but even with these smaller numbers the distinction between the polluted waters and the waters of good quality is more sharply marked than is the case with the numbers determined at the lower temperatures. Determinations at this temperature appear to be especially applicable to the control of water filters, since the relative purity of the raw and filtered waters, and the expression of the hygienic efficiency of such filters as the percentage removal of bacteria, are practically identical with similar determinations made at 20°, while the advantage obtained by having the results available within 24 hours would prove invaluable in controlling the operations of the filters and preventing any serious change in the character of the filtered water.

The numbers of bacteria determined at 40° C. are of great interest, since in this class of bacteria must be included the disease-producing organisms. The distinction between waters of different kinds and between waters of the same kind representing different degrees of pollution is well marked by counts at this temperature. The significance of the numbers of acid-producing bacteria determined at 40° C.—i. e., bacteria of the colon type—is well known. It may be said, however, that the usual practice of making qualitative determinations of the presence or absence of *B. coli* should be supplemented by quantitative determinations. It is the belief of the writer, based on experience gained in the present study, that considerable numbers of bacteria of the colon type may occur in waters which are supposedly quite pure, judging from their total bacterial content, while on other occasions a positive test for *B. coli* may be caused by an isolated organism of that type. In the one case the water would be open to suspicion, in the other case it would probbably be relatively harmless, although no such distinction could be made from the results of the qualitative tests.

The results of determinations of bacteria and of acid-producing organisms which are able to develop at a temperature of 50° C. are quite interesting. We see that relatively large numbers of bacteria of this type occur in sewages and in the effluents from sewage

filters in which the free passage of the sewage is practically unrestrained, while they are entirely absent from surface and ground waters which have not been exposed to any considerable pollution. It is rather surprising, however, that the numbers of bacteria of this type in very polluted Merrimack River water were not larger.

The information to be obtained by counts of bacteria and acid-producing organisms at any one of the above temperatures is greatly increased by the combination of the results obtained from counts at two or more temperatures, and this information is much more clearly shown if we express the relationship between the counts at the different temperatures methematically. Many individual differences between different waters are indicated by the bacterial ratios which would not be apparent in the results of the counts.

The writer is inclined to believe that a combination of counts at 20° C. and at 40° C. with corresponding ratios will yield information which will enable us to understand many of the hitherto unexplainable discrepancies in the results of bacterial analysis of water. The combination of 20° and 30° counts appears to be of less value while the value of 50° counts in combination with the other counts has not been sufficiently studied to determine their applicability.

In conclusion, the writer wishes to acknowledge his indebtedness to the various members of the laboratory force at the Lawrence Experiment Station for assistance in making the many determinations, and to Mr. H. W. Clark, chemist in charge of the station, whose hearty co-operation has made the investigation possible.

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THE TOXIC EFFECT OF CERTAIN ACIDS UPON TYPHOID AND COLON BACILLI IN RELATION TO THE DEGREE OF THEIR DISSOCIATION.*

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I. INTRODUCTION.

THE researches of the physical chemists, under the leadership of Arrhenius and Nernst, have shown that certain substances in aqueous solution become dissociated or broken up into electrically charged part-molecules (atoms or groups of atoms), which are called ions. The extent to which this occurs varies with different substances and is greatest in the most dilute solutions. With strong acids and bases, and their salts, it is practically complete at a strength of 0.001 normal. With such solutions it is evident that any effect, chemical or physiological, which they exert, must be due to the dissociated ions. The properties of a dilute solution of sodium chloride are the properties of sodium and chlorine ions, and the properties of hydrochloric acid, of hydrogen and chlorine ions. By the comparison of a series of properly selected compounds it is easy to determine the specific influence of each ion. The study of the toxic action of various substances in the light of these facts promises to be of great assistance in the development of a rational theory of disinfection.

The first definite statement of the relation between dissociation and disinfectant power with which we are familiar was made by Dreser. This author (Dreser, 1893), in a study of the pharmacological value of various salts of mercury, found that the double hyposulphite of mercury and potassium was much less poisonous than other compounds containing the same amount of mercury, and explained the phenomenon by the fact that this salt on dissociation does not set free mercury ions, but breaks up into potassium at the cathode and $Hg\ S_4O_6$ at the anode. His experiments were made on yeast cells, frogs, and fishes. In the former case he found it possible to prevent all development in a yeast culture by mercury salts, and

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then by the addition of potassium hyposulphite to permit fermentation without precipitating any of the mercury, simply by the formation of the differently dissociated double salt.

Scheurlen and Spiro (1897) confirmed the conclusions of Dreser as to the correlation between dissociation and disinfectant action among the mercury salts, and extended them to cover certain compounds of iron. At the same time they maintained that in other cases (the ethylchlorid and ethylsulphate of mercury) strong disinfectant action was apparently due, not to free ions, but to the undissociated molecule.

A number of phenomena which had long been empirically familiar in bacteriology found an easy explanation on the electrolytic theory of disinfection. The effect of temperature in increasing the activity of disinfectants, for example, had been pointed out by Koch (1881), and later by Behring (1890) and Heider (1892), and many others. It was at once obvious that this might be due in some cases to the increased dissociation at high temperatures. It would be well worth while today to see how far the increased activity of disinfectant runs quantitatively parallel to its dissociation. Again, Minervini (1898), and other investigators, have shown that various antiseptic agents (carbolic acid, chromic acid, mercuric chloride, and silver nitrate in Minervini's experiments) are much less active in alcoholic than in aqueous solutions. This fact, too, is easily explicable as due to the diminished dissociation in such solvents.

The relation between dissociation and toxicity was put upon a sound quantitative basis by the work of Krönig and Paul, first published in 1896 (Paul and Krönig, 1896), and in fuller detail in the next year (Krönig and Paul, 1897). These authors carried out an elaborate series of experiments on the disinfectant action of various salts, bases, and acids in the light of the new conclusions of physical chemistry. The details of the investigation were arranged with the greatest care in order to secure comparable results. Spores of *Bacillus anthracis* and vegetative cells of *Micrococcus aureus* were used, dried on Bohemian garnets. By using a definite number of garnets of a certain size shaken up with a suspension of an agar culture, after filtering through paper, and carefully drying, it was found possible to expose approximately the same number of cells in each experiment.

The garnets were dried for 12 hours at 7° and exposed in platinum sieves to the action of the disinfectant solution studied. The temperature was kept constant at 18° C. during the experiment, and after the desired time had elapsed the excess of disinfectant was carefully removed by appropriate reagents (neutralization of acids and bases, precipitation of heavy metals with ammonium sulphide, etc.). After thorough rinsing, the garnets were shaken up with water to detach the cells, which were then plated on agar. No attempt was made accurately to fix a killing point by testing a long series of dilutions of each disinfectant, and no exact calculations were made of dissociation. In a general way, however, the number of spores of *B. anthracis* which developed after treatment for a given time varied inversely with the amount of dissociation. Thus in the study of metallic salts it appeared that the activity of various compounds of mercury, silver, copper, and gold was greatest in those actively dissociated, and decreased in those which yield less free metallic ions. Solutions of mercuric chloride and silver nitrate, in alcohol, where no dissociation occurs, showed almost no disinfectant action. Furthermore, the toxic action of a salt having poisonous metallic ions was markedly diminished by the presence of a non-toxic salt of the same acid. This is in accord with physico-chemical theory; in any solution the ratio between the undissociated molecules and the product of free anions and cations is constant, so that the addition of sodium chloride to mercuric chloride keeps the proportion of chlorine ions the same, but replaces a portion of the mercury ions by those of sodium. So it appeared in Krönig and Paul's work that successive additions of sodium chloride to mercuric chloride progressively increased the number of colonies developing after the usual treatment. In the study of different salts of the same metal it was found that the acid radical may also exercise considerable influence on the disinfecting power.

With bases Krönig and Paul found the same general relation to hold, ammonium hydroxid, which is weakly dissociated, being a much less active disinfectant than the corresponding compounds of potassium, sodium, and lithium. The authors noted in a general way a diminution of disinfectant action in the presence of organic compounds. The decrease was most marked with the halogens and

oxidizing agents, and less with acids and bases. Disinfectants themselves of organic nature were least affected.

The particular phase of the subject with which we are especially concerned, the disinfectant action of the acids, was not exhaustively treated in this investigation. One series of experiments was made with normal and half-normal solutions, in which it was found that hydrofluoric, nitric, and trichloracetic acids in normal strength killed all the anthrax spores in 120 minutes. Normal chloric, hydrobromic, and hydrochloric acids and half-normal oxalic acid left a few spores alive after eight hours. Normal sulphuric acid was a little less effective, and normal phosphoric, formic, and acetic acids left large numbers of organisms alive after eight hours. Hydrocyanic acid in normal strength showed little action even after 30 hours. The investigators conclude that there is a general relation between the action of the acids and the amount of dissociated hydrogen present; but there appear many exceptions to a strict parallelism. The authors attribute these exceptional effects to the anion or the undissociated molecule, and point out that in more dilute solutions they tend to disappear. Thus, 0.06 normal solutions of hydrochloric, chloric, nitric, and trichloracetic acids showed about the same disinfectant action, apparently due to the presence of an approximately equal amount of dissociated hydrogen.

At a still earlier period a somewhat similar series of investigations to those of Paul and Krönig had been carried out in another field. This was a study of the relation between toxicity and dissociation as measured by the effect of various salts and bases, and a long series of organic and inorganic acids, on the higher plants, by Kahlenberg and True (1896). Their method consisted in determining the maximum strength of solution in which seedlings of *Lupinus albus* could grow. The seedlings were exposed for 15 to 24 hours, and their condition determined by their general appearance and by the growth which had taken place. These plants proved very sensitive to the action of dilute acid, a strength of from 0.00008 to 0.00064 normal killing them in almost every case. It is interesting to note that boric acid was endured in 10 times this strength. In general, the poisonous action ran parallel with the degree of dissociation, but certain of the organic acids showed relations of their

own. The authors concluded that "in the case of plants the toxic action of solutions of electrolytes, when dissociation is practically complete, is due to the action of the ions present. When dissociation is not complete, the undissociated part of the electrolyte may also exert a toxic effect." Heald (1896) extended the work of Kahlenberg and True to the seedlings of three other flowering plants, and reached the same general conclusions. All these authors pointed out clearly that the effect of the common mineral acids is due to hydrogen alone, since their anions have almost no strong toxic action when neutral salts are used.

The next work along these general lines was carried out in another field of botany by Stevens (1898), at the University of Chicago, the measure of viability used being the germination of fungus spores. A study had been made by Wüthrich (1892), at a much earlier date on the toxicity of metallic salts and acids for the spores of fungi; and Maillard (1898 and 1899) at about the same time reported experiments on the inhibition of the growth of *Penicillium* by copper salts. In Stevens' experiments the spores were inoculated into hanging-drop preparations of the solutions tested, and examined for development after 24 hours. The five organisms used exhibited marked differences in their susceptibility, although all were much less affected than the phanerogamous seedlings, requiring a strength of 0.01–0.02 normal acid to inhibit germination. The relative toxic effect of various substances was not unlike that observed by Heald and Kahlenberg and True. Mercuric chlorid and various copper salts proved most fatal, the acids and cyanides being less active. By the comparison of various substances it appeared that of the anions, CN, CrO_4, Cr_2O_7, and OH are poisonous, and of the cations, Hg, Cu, and H, while the halogens and SO_4 in dilute solutions exert no influence.

A still more exhaustive study of the effect of toxic agents upon the fungi was made by Clark in the next year (Clark, 1899). This investigator followed the same general method as that of Stevens, exposing spores in hanging-drop cultures to the activity of the agents to be tested. The cultures were divided into four classes: those which grew normally, those which showed irregular or retarded growth, those which failed to develop in the medium tested, but grew after

transfer to fresh beef infusion, and those which entirely succumbed to the action of the toxic substances. The wide difference between the concentration of acid producing, respectively, injury, inhibition, and death was one of the most interesting results of these experiments. As in Stevens' work, it was apparent that the fungi are extremely resistant to disinfectants, and it was necessary to use somewhat concentrated solutions, from 0.008 to 0.287 normal acid, for killing. It is perhaps partly for this reason that, as the author says, "in this study no new evidence has been adduced supporting the theory that the chemical activities of a substance are due wholly or chiefly to the ionized portion." On the other hand, it was held that "in the case of several acids, ionization lessens the chemical activities toward the substances involved in the life-processes of the plant." This conclusion is based on calculations of the specific toxicity of each ion and molecule, obtained by comparing the effects of different compounds varying from each other in one element or group. Thus, hydrochloric acid in the solutions used was over 90 per cent dissociated and since experiments with the similarly dissociated chloride of potassium showed this salt to be practically non-toxic, it is evident that its action was due to the combined effect of the hydrogen ion and the undissociated molecule. Nitric acid, dissociated in the same proportion was much more toxic. Since hydrogen ions are equal in the two cases and since the NO_3 ion is harmless, as shown by experiments with neutral salts, the increased effect must be due to the undissociated part of the molecule of nitric acid. Clark calculates that the toxic value of one molecule of undissociated HNO_3 is 7.7 times that of an ion of hydrogen, so that the acid actually loses nearly seven-eighths of its disinfectant power by becoming ionized.

The effect of sulphuric acid was about the same as that of hydrochloric; since it is less dissociated, the author attributes an appreciable influence to the anion, HSO_4. Acetic acid, at the strength used, is only 2 per cent dissociated, so that its high toxic effect is due to the un-ionized molecule.

The results obtained in this series of experiments with hydrocyanic acid are also interesting. Krönig and Paul (1897) found this acid almost without effect on anthrax spores, while Kahlenberg and True (1896), on the other hand, record very strong toxic action

on the seedlings of higher plants. In Clark's experiments it proved far more fatal than any other acid, being 70 times as active as hydrochloric. The molecule at the concentrations used is probably only slightly dissociated.

In some ways the most important work upon this subject was the very careful study made by Bial, of the antiseptic action of the hydrogen ion of dilute acids upon yeast. He first became interested in the problem from a consideration of the causes which allow production of gas in the stomach, and carried out his earliest experiments by observing the gas formation in yeast cultures in the presence of various substances present in the normal gastric juice. This series of studies (Bial, 1897) showed that the presence of albuminoid substances or of sodium chloride effected a marked restriction of the antiseptic action of hydrochloric acid. Bial at this time did not apply physico-chemical theories to the explanation of these phenomena; but in another contribution he made a fuller study of the problem. His later experiments (Bial, 1902) were again made with yeast cells, cultivated in fermentation tubes filled with grape-sugar solution to which various amounts of acid had been added; the antiseptic action was inversely registered by the amount of gas produced. The advantage of this method is its great delicacy; the fermentative power of the yeast responds to such extremely minute quantities of acid that the ionic effects are not complicated by other actions which appear in stronger solutions. Bial did not make exact calculations of the amount of dissociated hydrogen necessary to inhibit the yeast, but he found that a general relation existed between the ionization and the antiseptic action. The strongly dissociated acids — hydrochloric, sulphuric, nitric, and trichloracetic—entirely stopped the action of the yeast in concentrations of between 0.005 and 0.008 normal. Acids of an intermediate grade—phosphoric, formic, and oxalic—accomplished the same effect when 0.01 normal; while acids still less dissociated—acetic, benzoic, and butyric—stopped all fermentation only when 0.04 to 0.07 normal. The most striking feature of Bial's work was a series of experiments on the diminution of the antiseptic action of acids by the addition of neutral salts whose action is to decrease the dissociation of the acidic hydrogen. A solution of 0.01 normal formic acid and 0.3

normal sodium formate showed active fermentation, as did a solution of 0.0166 normal hydrochloric acid and 0.2 normal sodium chloride. The same phenomenon was observed with oxalates, nitrates, sulphates, and acetates. An exhaustive study in the case of hydrochloric acid showed that, while a certain amount of sodium chloride diminished the toxicity of the acid, a much larger amount actually increased it. Bial attributes this to a catalytic action of chlorine ions, but it seems to us that the facts may be explained more simply by the direct inhibiting effect of the sodium chloride and its ions. Bial found that twice normal sodium chloride without any acid prevented fermentation; and it is quite possible, in dealing with living organisms, that the combined effect of the acid and the chloride would be inhibitory at concentrations which with either acid or base alone might allow fermentation to go on. Bial studied also the effect of hydrochloric acid and sodium chloride in the presence of peptone, and found that the yeast would bear more of the salt than in the presence of acid alone.

The experiments of Paul and Krönig demonstrated clearly that in certain solutions disinfectant action runs parallel with the presence of dissociated ions. The work upon the higher plants and the mold fungi confirmed these results, but showed that in other cases the undissociated molecule is of great importance. Bial's studies brought out clearly the influence of neutral bodies, inorganic salts or proteids, in diminishing disinfectant action by decreasing dissociation.

The problem is, of course, complicated by still other chemical interactions which are more obscure. For example, Scheurlen, (1895), Beckmann (1896), Römer (1898), and Spiro and Bruns (1898) have shown that in the case of phenol and certain other organic disinfectants the addition of sodium chloride greatly increases toxic action. Still another factor which affects disinfectant power has been brought out in recent years by Nägeli (1893), and other observers—the presence of suspended solid particles of neutral character. In the most recent communication upon this subject by True and Oglevee (1905) it was shown that the toxic effect of metallic salts upon *Lupinus* may be entirely counteracted by the presence of finely divided particles of sand, glass, filter paper, coal, starch, or paraffin. On the other hand,the toxic effect of organic disinfectants

—phenol, thymol, and resorcinol—was affected only to a barely appreciable degree. The action when it occurs is explained by the power of suspended particles to remove dissolved substances by a process of adsorption, and the possibility suggests itself that the removal of ions by large organic molecules in true or colloidal solutions may be of an analogous character. Whatever the cause, this phenomenon must prove of far-reaching importance in bacteriology. Such facts as the observed multiplication of bacteria, when water samples are stored in glass bottles, may be the result of a removal of inhibiting substances by the adsorptive action of glass surfaces.

A considerable body of evidence in the field of animal physiology bears out these conclusions obtained from the study of bacteria and other plants. A fairly full summary of this literature may be found in the reviews of Cohen (1903) and Hamburger (1904). The work of Kahlenberg (1898) and other observers, who have shown that the taste of dilute solutions is in many cases due to the specific properties of the dissociated ions, is of interest. Studies which have been frequently cited were made by Loeb (1897 and 1898), and recently reprinted (Loeb, 1905), on the influence of free ions upon frog's muscle. The gastrocnemius muscle absorbs water and increases its weight in the presence of slight traces of acids or alkalis, and Loeb concluded that for the inorganic acids and bases this increase in weight is solely a function of the number of hydrogen and hydroxyl ions in the unit of volume. This sweeping conclusion is hardly borne out by his experiments. With the organic acids there was no relation whatever. Trichloracetic acid, almost entirely dissociated, and lactic acid, with only 11 per cent dissociation, gave practically the same results. With a series of 11 different organic acids of every degree of dissociation, the individual variation in weight-increase, with 0.009 normal solutions, ranged only between 3.9 per cent and 7.2 per cent.

A very significant line of physiological investigation concerns the binding of free ions by organic molecules of large size. In one of the most recent communications on this subject, Stiles and Beers (1905) have shown that the effect of calcium chloride, barium potassium chloride, and sodium nitrite upon plain, cardiac, and striped

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muscle of the frog, terrapin, and guinea-pig was reduced from one-half to three-fourths in the presence of white of egg, partially dialyzed serum, peptone, or starch. In some cases a combination between the inorganic body and the proteid has been demonstrated by freezing-point determinations, but in other cases particularly with the neutral salts, this has not been shown. As the authors suggest, these experiments point to the existence of "*physiological compounds* which are not demonstrable at all by chemical methods, but only by the reactions of living tissues."

2. OBJECT AND METHODS OF THE PRESENT INVESTIGATION.

The present investigation was begun with the intention of determining the effect of acid wastes in sewage upon the viability of the typhoid bacillus under practical conditions. It soon appeared, however, that the problem was too complex to be attacked in any general way without the preliminary determination of certain of the individual factors involved, under definitely controlled conditions. We have therefore attempted to find the disinfectant power of two mineral acids and two organic acids upon the typhoid bacillus in tap water and in the presence of peptone, and have controlled these experiments by a parallel series with the colon bacillus. The results, besides their specific value as determinations of the reactions of these two organisms to dilute acids, have a certain interest in relation to the general theory of disinfection. In all the experiments reviewed above, except Bial's, the acids used were tested in only a few widely differing strengths, so that the parallelism between disinfectant action and dissociation was not established with any great exactness. In the work of Krönig and Paul on anthrax spores and the various studies on the mold fungi, it was necessary to use such strong solutions that ionic effects were largely masked by the influence of the undissociated molecule, and in the studies of Kahlenberg and True and Heald on the phanerogams it was evident that with such complex organisms many other factors than the direct effect on protoplasm come into play. There was room, therefore, for a series of experiments on organisms sensitive to very dilute acids, carried out in sufficient detail to show definitely the relations between toxicity and dissociation.

With the view of securing exact quantitative results, we adopted the method of exposure to the acid tested, in a suspension from which samples were directly plated. This process has the obvious defect of permitting a certain amount of the disinfectant to be carried over to the plate, where it may exert an antiseptic action. We have really measured the combined disinfectant action in the suspension and possible antiseptic action in the plate. The action of organic matter in decreasing toxicity, shown by our experiments, must greatly reduce any such action in the plate.

The procedure in each experiment was as follows: A series of bottles, each containing 100 c.c. of sterile water (or peptone solution), was arranged in a row, and to each bottle was added a different amount of standardized acid from a graduated pipette. The amount of water in each bottle was measured at the end of the experiment, in order to obtain the exact strength of the solution. Immediately after the addition of the acid there was added to each bottle 1 c.c. of a fresh aqueous suspension of the bacteria tested. After standing for 40 minutes, lactose agar plates were made in duplicate, from the acidified bottles, and from controls with no acid. Colonies were counted after 24 hours' incubation at 37° C.

Forty minutes was selected, after some preliminary experiments, as the best period of exposure to the acid, since it gave sharper results than a shorter time. In the tests reported in the accompanying tables there was not a variation from the 40 minutes of more than one minute in most of the samples. The series for *B. typhi* in water, with HCl, were also examined after 100 minutes, and after 24 hours. In the sample containing 48 parts per million of sulphuric acid, there was, after 40 minutes, 59.3 per cent removal of *B. typhi;* after 100 minutes, 88.15 per cent removal; after 24 hours, 100 per cent removal. The sample containing 92.9 parts per million of sulphuric acid removed after 40 minutes 92.97 per cent; after 100 minutes, 99.99+ per cent; after 24 hours, 100 per cent. The removal was 100 per cent in all of the samples containing larger amounts of acid after 103 minutes, and in all of the samples after 24 hours.

The temperature factor was of considerable importance, and care was taken to keep conditions uniform. No agar was poured with a temperature greater than 50° C. It was found that a rise in tem-

perature in the presence of the acid was very fatal in its effect on the bacteria.

The typhoid culture used was obtained from the Massachusetts General Hospital, where it had been isolated from the spleen of a clinically typical case of typhoid fever; the colon bacillus was isolated in the laboratories of the Institute, and both gave all characteristic reactions. Twenty-four-hour agar-slant cultures were used in all cases.

The tables have been prepared to show, in the first column, the parts per million of the acid, and in the second column the parts per million of acidic hydrogen or, more accurately, of replaceable hydrogen. The third column shows the strength converted into terms of normality. The percentage dissociation of the acids at each dilution is given in the fourth column, and the actual parts of dissociated hydrogen in the fifth. The last two columns show the initial number of bacteria used as shown by blank controls and the percentage reduction after 40 minutes.

The tables show in general that with increasing quantities of disinfectant the bacterial reduction proceeds rapidly up to a certain point. After 99 per cent of the organisms have been killed, however, it takes a very considerable further increase of acid to produce sterilization. This is a point of very fundamental importance, and one which has been observed in studying the effect of such various agents upon the bacteria, that it deserves special attention. Sedgwick and one of us (Sedgwick and Winslow, 1902) have called attention to the persistence of a few specially resistant individuals when typhoid bacilli are exposed to the action of cold. After 14 days of exposure to freezing temperature 99.8 per cent of the organisms were killed, but after three months a few still survived. Johnson's tables of the reduction of typhoid and colon bacilli by copper salts (Johnson, 1905) show the same phenomenon, although he does not comment upon it specifically. More recently, Frost and Swenson (1906), and Gage and Stoughton (1906), have emphasized this peculiar phenomenon, in connection with resistance to high temperatures. The former authors, working with *B. dysenteriae*, found that "the majority of the cells were killed between 55° and 60°, but that frequently a relatively small number, possibly one individual

in a hundred thousand or a million, may persist at much higher temperatures, even 70°." Gage and Stoughton in their conclusions point out that "the great majority of the bacteria in any *B. coli* cultures are destroyed by five minutes' exposure to some temperature between 50° and 60° C. A few individuals, however, in each culture will survive much higher temperatures, in some cases remaining alive after exposure to 90° C. The very close range (about 10° C.) of temperature at which the destruction of the majority of the individual bacteria occurred, as compared with the considerable range (about 35° C.), in the temperatures at which complete sterilization was effected would indicate that the determination of the majority death-point would be of more value in species identification than is the determination of the absolute thermal death-point as at present employed."

Altogether it seems clear that among what are ordinarily considered non-sporing bacteria there exists a small proportion of individuals having specially high resistant powers against unfavorable conditions. The absolute death-point for these resistant forms is difficult to determine accurately on account of their small numbers and the consequent chances that they may be overlooked. We are inclined from our experience to agree with Gage and Stoughton as to the superior value for many purposes of the majority death-point (99 per cent), and we shall lay special stress on this in interpreting our results.

3. THE DISINFECTANT ACTION OF HYDROCHLORIC ACID AND SULPHURIC ACID UPON *B. TYPHI* AND *B. COLI*.

Hydrochloric acid and sulphuric acid were chosen as types for the study of strong mineral acids, and the experiments were carried out as described above. The water used was Boston tap water, containing before sterilization about 40 parts per million of residue, 15 parts of hardness, 0.015 part of free ammonia, and 0.144 part of albuminoid ammonia. The results are shown in Tables 1–4.

The 99 per cent killing-point with the hydrochloric acid is reached at a strength of 0.0077 normal; with 7.49 parts of dissociated hydrogen per million, and the absolute killing-point, as nearly as it can be determined, with a 0.0123 normal solution containing 11.80 parts

TABLE 1.

ACTION OF HYDROCHLORIC ACID ON *B. coli* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
38.1	1.05	0.0010	98.0	1.03	40,000	00.00
77.6	2.13	0.0021	98.0	2.09	"	45.00
104.5	3.32	0.0033	97.5	3.24	"	72.85
149.0	4.08	0.0041	97.3	3.98	"	82.50
178.9	4.90	0.0049	97.2	4.76	"	97.50
281.0	7.71	0.0077	97.0	7.49	"	99.87
298.0	8.17	0.0082	96.5	7.90	90,000	99.96
377.0	10.61	0.0106	96.4	10.24	"	99.99
447.0	12.26	0.0123	96.4	11.80	"	100.00
515.0	.....	.....	96.3	....	"	100.00
590.0	.....	.....	96.3	....	"	100.00
663.0	.....	.....	96.2	....	"	100.00

TABLE 2.

ACTION OF SULPHURIC ACID ON *B. coli* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment.	Percentage Reduction of Bacteria after 40 Min.
45.9	0.94	0.0009	93	0.87	60,000	66.20
46.5	0.95	0.0009	93	0.87	140,000	80.00
111.5	2.28	0.0023	90	2.05	"	81.43
138.3	2.82	0.0028	89	2.51	60,000	86.50
176.6	3.62	0.0036	88	3.18	"	96.25
178.2	3.64	0.0036	88	3.20	140,000	82.14
225.2	4.59	0.0046	86	3.94	60,000	96.25
257.2	5.25	0.0052	85	4.46	"	98.50
311.5	6.36	0.0064	84	5.35	140,000	97.50
375.9	7.68	0.0077	83	6.38	"	98.86
470.0	9.60	0.0096	80	7.68	"	99.92
552.0	11.30	0.0113	79	8.93	"	99.92
630.0	12.90	0.0129	78	10.05	"	99.95
692.5	14.13	0.0141	77	10.90	"	99.95
812.0	16.57	0.0166	76	12.60	"	100.00
910.0	18.57	0.0186	75	13.95	"	100.00

of dissociated hydrogen. With the sulphuric acid the 99 per cent reduction was reached at a strength of 0.0096 normal, with 7.68 parts of dissociated hydrogen, and the 100 per cent reduction at a strength of 0.0166 normal, with 12.6 parts of dissociated hydrogen. These results show a direct relation between disinfectant action and free hydrogen ions. The normal strengths of the killing solutions do not correspond very closely; 0.0077 sulphuric acid failed to do what the same strength of hydrochloric acid did, and 0.0129 and 0.0141 sulphuric acid failed to do what 0.0123 hydrochloric acid did. On the other hand, when we compare dissociated hydrogen, allowing for the greater ionization of the hydrochloric acid, the discrepancies disappear. The same concentrations of dissociated hydrogen, within

the limits of accuracy of the experiment, produced respectively the 99 per cent and the 100 per cent reduction in the two acids.

TABLE 3.

ACTION OF HYDROCHLORIC ACID ON *B. typhi* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
38.2	1.00	0.0010	98.2	1.02	7,050	79.20
75.3	2.06	0.0021	97.9	2.02	"	88.10
109.5	3.00	0.0030	97.8	2.94	"	99.30
148.2	4.06	0.0041	97.3	3.95	"	99.86
182.5	5.00	0.0050	97.1	4.85	"	100.00
216.8	5.92	0.0059	....	....	"	100.00
248.0	6.80	0.0068	....	....	"	100.00
275.5	7.54	0.0075	....	....	"	100.00

TABLE 4.

ACTION OF SULPHURIC ACID ON *B. typhi* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1 000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
48.0	0.98	0.0010	93.1	0.91	135,000	59.30
92.9	1.88	0.0019	90.5	1.70	"	92.97
139.4	2.84	0.0028	89.5	2.54	"	99.99
188.9	3.85	0.0038	87.0	3.35	"	96.10
223.0	4.55	0.0045	85.5	3.90	"	100.00

Tables 3 and 4 show that the typhoid bacillus is considerably more sensitive than the colon bacillus in its reaction to an excess of acid. The 99 per cent reduction was reached with hydrochloric acid at a strength of 0.0030 normal and 2.94 parts of dissociated hydrogen, and the 100 per cent reduction with 0.0050 normality and 4.85 parts of dissociated hydrogen. With sulphuric acid the 99 per cent reduction was reached with 0.0028 normality and 2.54 parts of hydrogen, and the 100 per cent reduction with 0.0045 normality and 3.90 parts of hydrogen. The fact that the 0.0038 normal solution of sulphuric acid showed only 96 per cent reduction is one of the abnormalities which unfortunately sometimes occur in bacteriological work. In general, the results show again that the two acids exert the same quantitative effect, although in this case, the solution being weaker and the dissociation of the two acids more nearly the same, the difference between normal strength and concentration of dissociated hydrogen is not clearly shown.

The critical points derived in these tests are brought together in Table 5. They show that the typhoid bacillus is a little less than half as resistant as the colon bacillus to dilute acids, and that the toxicity of these acids depends, not on their normal strength of acid or on the kind of acid used, but on the number of dissociated hydrogen ions. Between 7.4 and 7.7 parts of dissociated hydrogen effects a 99 per cent reduction of the colon bacillus, and between 11.8 and 12.6 parts, a 100 per cent reduction. For the typhoid bacillus the corresponding figures are 2.5–3.0 parts and 3.9–4.9 parts. Since at the dilutions used the hydrochloric acid was over 96 per cent dissociated, its effect must have been almost entirely ionic; and since the sulphuric acid at 75 per cent dissociation showed only the toxicity which would have been expected from its dissociated hydrogen, it appears that in this case too the undissociated molecule exerts no appreciable influence. The anions have been shown to be neutral in the experiments of other observers. It is evident, then, that the toxicity of these acids at high dilution is a function of the dissociated hydrogen.

TABLE 5.

DISINFECTANT ACTION OF MINERAL ACIDS IN TAP WATER.

	B. coli				*B. typhi*			
	99% Reduction		100% Reduction		99% Reduction		100% Reduction	
	HCl	H_2SO_4	HCl	H_2SO_4	HCl	H_2SO_4	HCl	H_2SO_4
Normality	0.0077	0.0096	0.0123	0.0166	0.0030	0.0028	0.0050	0.0045
Parts per 1,000,000 dissociated hydrogen	7.49	7.68	12.80	12.60	2.94	2.54	4.85	3.90

4. THE DISINFECTANT ACTION OF ACETIC ACID AND BENZOIC ACID UPON *B. TYPHI* AND *B. COLI*.

We next desired to study examples of the incompletely dissociated organic acids. Acetic and benzoic acids were selected as types, and the experiments were carried out as before. The results obtained with benzoic acid are probably somewhat inaccurate on account of the difficulty of securing complete solution. The results are shown in Tables 6–8.

An inspection of these tables shows a marked difference from the results obtained with the mineral acids. With *B. coli* in acetic acid the 99 per cent reduction is reached at a strength of 0.0812 normal, and

TABLE 6.

ACTION OF ACETIC ACID ON *B. coli* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
111.3	1.85	0.0018	....	....	60,000	00.00
333.0	5.54	0.0055	6.40	0.35	"	00.00
552.0	9.18	0.0092	4.50	0.41	"	16.67
732.0	12.20	0.0122	3.50	0.43	"	23.33
1,095.0	18.25	0.0182	3.05	0.56	"	38.33
1,386.0	23.20	0.0232	2.75	0.64	"	51.67
1,825.0	30.41	0.0304	2.45	0.75	"	55.00
2,260.0	37.70	0.0377	2.20	0.83	"	56.67
3,081 0	51.40	0.0514	1.90	0.98	"	58.33
3,698.0	61.70	0.0617	1.70	1.05	"	63.33
4,800.0	80.20	0.0802	1.50	1.20	"	91.00
4,875.0	81.25	0.0812	1.50	1.21	90,000	99.99
5,610.0	93.50	0.0935	1.35	1.26	"	100.00

TABLE 7.

ACTION OF BENZOIC ACID ON *B. coli* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
237.9	1.95	0.0019	16.0	0.31	70,000	00.00
337.0	2.76	0.0028	13.6	0.38	"	50.00
406.0	3.33	0.0033	12.5	0.41	"	60.75
675.0	5.54	0.0055	9.3	0.52	"	67.80
1,184.0	9.72	0.0097	7.5	0.73	"	99.99
2,425.0	19.90	0.0199	5.4	1.07	100,000	100.00

TABLE 8.

ACTION OF BENZOIC ACID ON *B. typhi* IN TAP WATER.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
29.23	0.24	0.0002	40.0	0.10	50,000	44.00
140.30	1.15	0.0011	20.3	0.23	"	54.00
243.80	2.00	0.0020	15.4	0.31	"	62.00
333.00	2.73	0.0027	13.6	0.37	"	70.00
427.00	3.52	0.0035	10.4	0.37	"	92.00
689.00	5.75	0.0057	9.8	0.56	"	100.00
1,292.00	10.60	0.0106	7.2	0.76	"	100.00

the 100 per cent reduction at 0.0935 normal. The acid at these strengths is only a little over 1 per cent dissociated, and the amount of dissociated hydrogen present a little over 1.2 parts per million. Since this is only about one-sixth the strength of ionic hydrogen necessary to produce similar results with the mineral acids, it is evident that the toxic action of the acetic acids is due chiefly to the anion or the undissociated molecule, the latter, being so much greater in

amount, probably playing the principal part. The same thing is true of benzoic acid. Here the molecule is more highly toxic, producing a 99 per cent reduction at 0.0097 normality and a 100 per cent reduction at 0.0199 normality, with about 1 per cent dissociation. As in the case of the mineral acids *B. typhi* is more sensitive than *B. coli*, showing 100 per cent reduction with benzoic acid at a strength of 0.0057 normal.

It appears, then, that the toxicity of these organic acids is due not mainly to hydrogen ions, but to the action of the undissociated molecule, varying widely, as might be expected, with the acid employed. A comparison of the corresponding toxic normal

TABLE 9.

TOXICITY OF ORGANIC AND MINERAL ACIDS FOR *B. coli* AND *B. typhi*. STRENGTH IN NORMALITY PRODUCING 99 PER CENT AND 100 PER CENT REDUCTION.

	B. coli		*B. typhi*	
Acid	99% Reduction	100% Reduction	99% Reduction	100% Reduction
Hydrochloric	0.0077	0.0123	0.0030	0.0050
Sulphuric	0.0096	0.0166	0.0028	0.0045
Acetic	0.0812	0.0935	.....	.....
Benzoic	0.0097	0.0199	.....	0.0057

strength, made in Table 9, shows that benzoic acid is almost as toxic as the mineral acids, the effect being due in one case to the whole molecule, and in the other case to hydrogen ions. Acetic acid, on the other hand, has only 10–20 per cent as high a disinfectant action.

5. THE DIMINISHED TOXICITY OF ACIDS IN THE PRESENCE OF PEPTONE.

Having fixed with some precision the killing-point for the various acids studied, when acting in tap water, we next desired to determine what would occur in the presence of organic matter. A series of experiments was carried out, parallel to those reported above, except that a 1 per cent solution of Witte's peptone was used instead of tap water. The results with the mineral acids, presented in Tables 10–12, showed that the toxicity of the acid is profoundly modified by the presence of organic matter.

TABLE 10.

DISINFECTANT ACTION OF HYDROCHLORIC ACID ON *B. coli* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
1,118	30.62	0.0306	94.8	29.0	90,000	40.00
1,825	50.00	0.0500	94.8	47.5	"	93.35
2,502	69.00	0.0690	93.0	64.2	"	97.97
2,950	80.80	0.0808	92.0	74.4	"	97.74
3,470	95.20	0.0952	91.5	87.5	"	99.98
3,685	97.80	0.0978	91.4	89.4	"	100.00
4,020	110.00	0.1100	....	....	"	100.00

TABLE 11.

DISINFECTANT ACTION OF SULPHURIC ACID ON *B. coli* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c. c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
970	.....	.....	....	....	140,000	00.00
1,204	.....	.....	....	....	"	00.00
1,405	.....	.....	....	....	"	00.00
1,536	31.4	0.0314	70.0	22.0	"	00.00
1,728	35.3	0.0353	68.5	24.2	"	20.00
1,962	40.1	0.0401	67.5	27.1	"	61.43
2,066	40.9	0.0409	67.0	27.4	"	64.29
2,399	48.9	0.0489	65.4	32.0	60,000	76.67
2,639	53.8	0.0538	64.8	24.9	"	79.17
2,912	59.4	0.0594	64.2	38.1	"	91.67
3,065	62.6	0.0626	63.2	30.6	"	93.33
3,258	66.5	0.0665	62.8	41.8	"	94.17
3,450	70.4	0.0700	61.9	43.5	"	98.85
4,610	94.2	0.0942	58.9	55.5	65,000	99.99
5,298	108.1	0.1081	57.0	61.6	"	100.00
6,555	135.2	0.1352	....	....	"	100.00
7,800	159.3	0.1593	....	....	"	100.00

TABLE 12.

DISINFECTANT ACTION OF HYDROCHLORIC ACID ON *B. typhi* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
1,107	30.3	0.0330	94.8	28.7	50,000	99.99
1,740	47.6	0.0476	93.8	44.6	50,000	100.00

In these tables the dissociation values given are those determined for distilled water, and not those which actually obtain in a peptone solution. The amount of dissociated hydrogen required for disinfection, when estimated in this way, is seen to be nearly 10 times as great as in tap water. For comparison the results are brought together in Table 14.

TABLE 13.

DISINFECTANT ACTION OF SULPHURIC ACID ON *B. typhi* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c. c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
819	17.03	0.0170	74.5	12.7	85,000	00.00
960	20.00	0.0200	73.7	14.7	"	17.65
1,104	22.52	0.0225	72.0	16.2	"	17.65
1,130	23.17	0.0232	72.0	16.7	"	34.10
1,288	26.32	0.0265	71.0	18.7	"	61.20
1,453	29.63	0.0296	70.2	20.8	28,000	82.15
1,472	30.03	0.0300	70.1	21.0	85,000	76.45
1,519	31.66	0.0317	69.7	21.6	28,000	89.30
1,587	32.39	0.0324	69.5	22.5	"	89.30
1,612	32.89	0.0329	69.0	22.7	"	100.00
1,678	34.24	0.0342	68.5	23.4	"	97.86
1,879	38.32	0.0383	68.0	26.0	"	100.00
1,994	.....	.....	....	....	"	100.00
2,045	.....	.....	....	....	"	100.00
2,115	.....	.....	....	....	"	100.00
2,119	.....	.....	....	....	"	100.00
2,399	.....	.....	....	....	"	100.00

TABLE 14.

COMPARATIVE TOXICITY OF MINERAL ACIDS IN DISTILLED WATER AND 1 PER CENT PEPTONE SOLUTION.

(Parts per 1,000,000 of dissociated hydrogen.)

	B. coli				*B. typhi*			
	99% Reduction		100% Reduction		99% Reduction		100% Reduction	
	HCl	H_2SO_4	HCl	H_2SO_4	HCl	H_2SO_4	HCl	H_2SO_4
Distilled water.....	7.49	7.68	11.80	12.60	2.94	2.54	4.85	3.90
1 per cent peptone.....	87.5	55.5	89.4	61.6	28.7	....	44.6	22.7

It is evident that in some way the peptone exerts a strong influence in counteracting the toxic effect of the acids. It at first occurred to us that this might be due simply to the fact that peptone solution furnished a more favorable medium for the bacteria, and thus enabled them to resist unfavorable conditions. Such an effect would, however, hardly be expected in so short a period as 40 minutes; and this explanation fails to account for the fact that the toxicity of the hydrochloric acid is much more diminished than that of the sulphuric acid. Reference to Tables 15–17, which show the results obtained with the organic acids, makes it still clearer that a specific chemical action is involved.

Evidently with the organic acids disinfectant power is much less affected by the presence of peptone. With *B. coli* acetic acid produces a 100 per cent reduction when in a strength of 0.0935

TABLE 15.

DISINFECTANT ACTION OF ACETIC ACID ON *B. coli* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
4,350	72.5	0.0725	1.60	1.16	50,000	00.00
4,750	79.3	0.0793	1.50	1.19	"	00.00
5,340	89.0	0.0890	1.40	1.25	"	25.00
5,975	97.0	0.0970	1.30	1.26	"	66.00
5,995	98.0	0.0980	1.30	1.27	"	61.72
6,861	114.2	0.1142	1.25	1.42	65,000	98.75
7,540	125.8	0.1258	1.15	1.45	"	93.30
8,360	139.5	0.1395	1.05	1.46	"	100.00
9,125	152.1	0.1521	1.03	1.57	"	100.00
10,475	174.5	0.1745	1.00	1.75	"	100.00
10,720	178.8	0.1788	1.00	1.78	"	.....
.....	210.0	0.2100	.....	....	"	.....
14,620	244.0	0.2440	0.90	2.20	"	100.00

TABLE 16.

DISINFECTANT ACTION OF BENZOIC ACID ON *B. coli* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
1,638	13.18	0.0132	6.4	0.84	70,000	28.6
1,720	14.11	0.0141	6.2	0.87	70,000	28.6
2,105	17.20	0.0172	5.8	1.00	100,000	0.0
3,365	27.60	0.0276	5.2	1.43	100,000	73.0
5,555	47.30	0.0473	3.2	1.51	100,000	100.0

TABLE 17.

DISINFECTANT ACTION OF BENZOIC ACID ON *B. typhi* IN 1 PER CENT PEPTONE SOLUTION.

Acid Parts in 1,000,000	Hydrogen Parts in 1,000,000	Normality	Per Cent of Dissociation	Dissociated Hydrogen. Parts in 1,000,000	Bacteria per c.c. before Treatment	Percentage Reduction of Bacteria after 40 Min.
1,434	11.72	0.0117	6.9	0.81	50,000	20.00
1,562	12.80	0.0128	6.6	0.84	50,000	20.00
2,155	17.80	0.0178	5.7	1.01	56,000	81.43
2,917	24.30	0.0243	4.9	1.19	56,000	100.00

normal. In the presence of peptone the required strength is 0.1395 normal. With *B. coli* the corresponding 100 per cent reduction strengths for benzoic acid are 0.0199 in water and 0.0473 in peptone solution. With *B. typhi* the respective strengths of benzoic acid are 0.0057 and 0.0243.

In a general way we may say that the presence of 1 per cent peptone solution diminishes the toxicity of hydrochloric acid, measured in terms of dissociated hydrogen, to from one-eighth to one-tenth its water value, and that of sulphuric acid to from one-fifth to one-eighth

its water value. The toxicity of benzoic acid, measured in normality, is diminished under the same conditions to from one-fourth to one-half, and that of acetic acid to a little over one-half its water value.

The most probable explanation of this phenomenon is the formation of a loose compound between the proteid molecules and the acids which would diminish the toxicity of the latter, just as Stiles and Beers (1905) have shown that such a combination alters the effect of mineral salts on muscle.

Bugarzky and Liebermann (1898), Cohnheim and Krieger (1900), and other observers, have proved the existence of such loose compounds between proteids and acids by freezing-point determinations. We desired, however, to examine the actual substance used in our own experiments. Through the kindness of Dr. Raymond Haskell, of the Research Laboratory of Physical Chemistry of this Institute determinations of electrical conductivity were made on the peptone solution used in our experiments, on a solution of hydrochloric acid in distilled water, and on a solution of the same strength in the peptone solution.

The specific conductivity of the peptone solution was 0.0004, showing that it was fairly pure. That of the hydrochloric acid solution 0.02 normal or 720 parts HCl per million, 90.46 per cent dissociated, was 0.007. The 1 per cent peptone solution containing 0.02 normal hydrochloric acid gave a conductivity of 0.002, showing that approximately four-fifths of the hydrochloric acid had been neutralized by the peptone.

It is evident that the effect of the peptone in decreasing the toxicity of the hydrochloric acid may be explained by the fact that the number of dissociated hydrogen ions is decreased by the peptone in the same degree. The effect would naturally be less marked, as we find to be the case, with sulphuric acid, since this acid is less ionized to start with. Finally, the un-ionized organic acids are least affected. The decreased toxicity which does occur with them may perhaps be due to a loose compound with their whole-molecule—what Stiles and Beers call a "physiological compound."

6. GENERAL CONCLUSIONS.

It appears from our experiments that the typhoid bacillus is highly sensitive to an excess of acid, being destroyed in an aqueous suspen-

sion by 40 minutes' exposure to a 0.005 normal solution of either hydrochloric, sulphuric or benzoic acid. The colon bacillus will endure exposure, under similar conditions, to solutions from two to four times as strong. Ninety-nine per cent of the bacteria in a suspension are killed by solutions of from one-half to two-thirds this strength, the last few organisms being especially resistant.

The mineral acids, hydrochloric and sulphuric, are fatal in concentrations at which they are highly dissociated. Their action runs parallel, not to their normal strength, but to the number of free hydrogen ions per unit volume. With the two organisms tested, both the 99 per cent and the 100 per cent reductions were affected, at the same concentration of dissociated hydrogen, whichever acid was used.

The organic acids, acetic and benzoic, are fatal to the typhoid and colon bacilli at a strength at which they are only slightly dissociated. The effect here appears to be due to the whole-molecule and is specific for each acid, acetic having only 10–20 per cent the toxicity of benzoic.

The presence of 1 per cent of peptone greatly diminishes the toxic action of acids, the action being somewhat less marked with sulphuric acid than with hydrochloric, and still weaker with the organic acids. In the case of hydrochloric acid we find that the diminished toxicity is accounted for by decreased ionization.

It is evident that the action of organic matter and other neutral substances in decreasing toxicity greatly complicates the study of disinfectant action. It will be necessary to bear this phenomenon in mind in considering the composition of culture media, since the apparent acidity, as determined by titration, may be quite different from the effective acidity which influences living organisms. With the mineral acids, any factor which affects dissociation, such as the presence of neutral salts of the same anion, will change the effective acidity. In considering the viability of disease germs in sewage and water, it is evident that differences in dilution and the effect of inorganic salts, organic matter, and suspended solids introduce such complex factors that detailed studies of specific local conditions are desirable.

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THE INHIBITING EFFECT OF CERTAIN ORGANIC SUBSTANCES UPON THE GERMICIDAL ACTION OF COPPER SULPHATE.*

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INTRODUCTION.

THE germicidal action of copper salts dissolved in water has frequently been found to depend largely upon the character of the water itself. Ellms (1905) has pointed out the influence of the hardness and turbidity of the water. Johnson and Copeland (1905) found that in a sewage effluent, to which a large number of typhoid organisms had been added, an amount of copper sulphate equal to a concentration of 20 parts of copper per million reduced the number of bacteria from 1,300,000 to 600 per cubic centimeter in 15 hours. In distilled water, other conditions being the same, the reduction was from 1,300,000 to 11. Kraemer (1905) and Basset-Smith (1905) have both shown that the toxic action of copper on the typhoid organism is much greater in distilled water than in tap water.

It is not difficult to determine the nature of the influence exerted by mineral impurities in the water. Dissolved carbonates bring about a direct precipitation of the copper. Even such insoluble material as kaolin has been shown by Sullivan (1905) to possess the power of reacting with copper salts, in some cases completely removing the copper from solution. True and Oglevee (1905) have confirmed the earlier results of Nägeli showing that adsorption often plays an important rôle, and that powdered glass or sand may destroy in large measure the toxic action of dilute metallic solutions.

In case of organic impurities the nature of the influence is not quite so clear. Direct precipitation of the copper may occur, especially in sewages. On the other hand, the presence of certain classes of organic matter, such as leaf infusion, has been shown to prevent the precipitation of copper by alkaline carbonates (Ellms, 1905). In such

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cases it must be assumed that certain organic compounds are formed. If such compounds are found upon investigation to be non-toxic, or to have a lower toxic value than the original copper salt, this fact may throw some light upon the nature of the toxic action itself.

It was the purpose of the present investigation to study the germicidal action of copper sulphate upon the typhoid organism in distilled water and in the presence of certain organic compounds. Organic substances were selected which would not in any case precipitate the copper, and which, according to the chemical evidence, do not form any direct union with it. This does not preclude the formation of a "physiological compound," as defined by Stiles and Beers (1905), namely, a compound which is not readily detectible by chemical means, but which possesses characteristic physiological properties. For this purpose dextrose and peptone were used. The organic matter occurring naturally in Boston tap water, a colored surface water, was also studied.

METHODS.

Preparation of copper sulphate.—The copper sulphate used was carefully prepared to assure a pure product. In particular was it desired to obtain salt free from ammonia, since there is reason to believe that the double or cuprammonium salt will have a distinctly different toxic effect from that of the simple copper salt. Some "C. P." copper sulphate crystals were dissolved in water, making about a 10 per cent solution. To this solution a small amount of Na_2CO_3 was added, and the solution was boiled for some time to expel the ammonia. It was then acidified and submitted to electrolysis with a small current, about 0.05 ampere, and a potential difference through the solution of about one volt. Copper was deposited on the inside of a platinum dish which served as a cathode. This deposit of copper was then washed with ammonia-free water and redissolved in dilute sulphuric acid by reversing the current. The product obtained by crystallizing this solution was recrystallized from ammonia-free water to free it from the last traces of acid. It was dried for several days over calcium chloride and caustic potash.

Preparation of potassium sulphate.—This salt was prepared by twice recrystallizing the best Merck preparation from ammonia-free water.

Tenth-molar (fifth-normal) solutions of these salts were made up. For use 1 c.c. of these solutions was diluted to 100 with ammonia-free water. In the following tabulated results all references to the salt solutions are to these dilute (N/500) solutions.

The organic compounds.—Witte's peptone and Merck's "C. P." dextrose were used.

The typhoid cultures.—The culture used (No. 2006) was one of those used in some earlier work. It was obtained from Dr. J. H. Wright, of the Massachusetts General Hospital. It was taken from the spleen at an autopsy on May 26, 1905. The clinical diagnosis was typhoid fever. The culture, according to Wright, gave all the ordinary tests for typhoid fever, including the Widal test.

It was submitted to the ordinary diagnostic tests, with the following results: fermentation tube with dextrose broth, growth in closed arm, acid produced, no gas; milk not coagulated, slight acid production; no indol from peptone; nitrates reduced slightly; gelatin not liquefied in two weeks; growth on agar slant, scanty, thin, translucent; it reacts with the sera of two rabbits into which two other strains of typhoid organisms had been introduced subcutaneously.

Cultures A, B, and C were obtained from plates made from Bottle 2 in Experiment 5. They had lived for 48 hours in the presence of a concentration of 0.63 parts of copper per million in distilled water. They gave all the above tests exactly as the original culture, except that they were not submitted to the Widal test.

Technique of the experiments.—The experiments were made under as uniform conditions as possible, and in the following manner:

In all cases, except where tap water is specified, sterilized ammonia-free water was used. When dextrose or other solutions were used, these were made up with ammonia-free water and sterilized. The organism was grown for 24 hours at 37° on the surface of an agar slant. A loop of the culture was then removed and placed in 100 c.c. of water. After thorough shaking, proper amounts of this suspension were added to the bottles of water in which the experiments were to to be made. These bottles were then thoroughly shaken, and the the bacteria determined in each by properly diluting 1 c.c. and plating. For plating agar was used, and all plates were grown for 15 hours at 37° C. The salt solution was then added. In the same manner

determinations of the numbers of organisms present were made at intervals during the experiment, as indicated in the tabulated results.

In the following tables the full details of each experiment are first given, including the actual bacterial counts. In order to compare the various experiments, the bacterial results have been calculated

EXPERIMENT 1.

STARTED JANUARY 24, 1906.

Bottle No.	Water (c.c.)	$CuSO_4$ Sol. (c.c.)	K_2SO_4 Sol. (c.c.)	Concent. of Salt (N/1,000,000)	Copper (Parts per Mill.)	Counts			
						Initial	5 Min.	64 Hrs.	24 Hrs.
1	98	0.6	...	12	0.38	380,000	125,000	6,000	42
2	98	1.0	...	20	0.63	400,000	45,000	1,700	12
3	100	2.0	...	40	1.26	350,000	25,000	300	9
4	98	...	0.6	12	....	370,000	.....	370,000	50,000
5	98	...	1.0	20	....	410,000	.....	460,000	45,000
6	99	...	2.0	40	....	400,000	.....	400,000	43,000

Temperature, 20°–22°.

EXPERIMENT 2.

TAP WATER—STARTED JANUARY 29, 1906.

Bottle No.	Water (c.c.)	$CuSO_4$ So. (c.c.)	K_2SO_4 So. (c.c.)	Concent. of Salt (N/1,000,000)	Copper (Parts per Mill.)	Counts					
						Initial	1 Min.	10 Min.	30 Min.	6 Hrs.	29 Hrs.
1	89	0.4	...	8.8	0.28	170,000	180,000	170,000	170,000	110,000	9,800
2	94	0.8	...	16.8	0.53	180,000	.....	.....	.....	50	510
3	84	1.2	...	28.0	0.88	190,000	.....	.....	.....	.....	100
4	90	2.0	...	44.0	1.39	150,000	.....	.....	.....	.....	80
5	88	3.0	...	66.0	2.08	180,000	120,000	100	100	50	0
6	88	...	0.4	8.8	....	140,000	.....	.....	.....	.....	240,000
7	89	...	0.8	18.0	....	160,000	.....	.....	.....	.....	336,000
8	90	...	1.2	26.0	....	180,000	.....	.....	.....	.....	92,000
9	95	...	2.0	42.0	....	170,000	.....	.....	.....	.....	69,000
10	92	...	3.0	66.0	....	200,000	.....	.....	.....	.....	80,000

Temperature, 20°–23°.

EXPERIMENT 3.

STARTED FEBRUARY 6, 1906.

Bottle No.	Water (c.c.)	$CuSO_4$ Sol. (c.c.)	K_2SO_4 Sol. (c.c.)	Concent. of Salt (N/1,000,000)	Copper (Parts per Mill.)	Counts			
						Initial	1 Hr.	6 Hrs.	24 Hrs.
1	100	0.5	...	10	0.31	125,000	.....	5,300	8
2	99	1.0	...	20	0.62	135,000	.....	700	6
3	105	2.0	...	40	1.24	140,000	.....	400	9
4	98	3.0	...	60	1.86	128,000	80,000	130	0
5	99	...	0.5	10	....	140,000	.....	120,000	80,000
6	97	...	1.0	20	....	136,000	.....	100,000	70,000
7	98	...	2.0	40	....	132,000	.....	100,000	63,000
8	98	...	3.0	60	....	140,000	.....	120,000	56,000

Temperature, 20°–22°.

into percentage of the initial numbers, and these results have been used in the discussion of the table.

In each case in which copper sulphate was used the control contained an equimolar amount of potassium sulphate. The controls therefore differed from the tests simply in the substitution of potassium for the copper.

EXPERIMENT 4.

STARTED FEBRUARY 13, 1906.

Bottle No.	Water		Dextrose (gm.)	$CuSO_4$ Sol. (c.c.)	K_2SO_4 Sol. (c.c.)	Concent. of Salt (N/1,000,000)	Copper (Parts per Mill.)	Counts				
	Kind	c.c.						Initial	3 Hrs.	6 Hrs.	23 Hrs.	48 Hrs.
1	Best	98	..	0.5	...	10	0.31	540,000	1,800	1,400	660	660
2	"	99	..	1.0	...	20	0.63	540,000	400	100	46	350
3	"	99	1	0.5	...	10	0.31	560,000	415,000	640,000	190,000	4,300
4	"	98	1	1.0	...	20	0.63	500,000	170,000	82,000	100	600
5	Tap	90	..	0.5	...	10	0.31	540,000	134,000	2,000	125	9
6	"	87	..	1.0	...	20	0.63	560,000	6,300	700	15	2
7	Best	98	..	...	1.0	20	....	500,000	.....	520,000	95,000	10,000
8	"	98	1	...	1.0	20	....	500,000	.....	490,000	200,000	420,000
9	Tap	90	..	...	1.0	20	....	540,000	.....	600,000	110,000	1,200

Temperature, 20°–24°.
"Best water" is ammonia-free, distilled water.

EXPERIMENT 5.

STARTED FEBRUARY 15, 1906.

Bottle No.	Water (c.c.)	Dextrose (gm.)	$CuSo_4$ Sol. (c.c.)	K_2So_4 Sol. (c.c.)	Concent. of Salt, (N/1,000,000)	Copper (Parts per Mill.)	Counts			
							Initial	6 Hrs.	24 Hrs.	48 Hrs.
1	100	..	0.5	...	10	0.31	30,000	600	184	40
2	100	..	1.0	...	20	0.63	26,000	200	70	23
3	99	1	0.5	...	10	0.31	26,000	1,300	1,800	900
4	100	1	1.0	...	20	0.63	38,000	contam	inated	
5	98	..	...	0.5	10	....	28,000	30,000	14,000	9,000
6	99	..	...	1.0	20	....	30,000	27,000	10,000	.....
7	99	1	...	0.5	10	....	24,000	25,000	12,000	.....
8	98	1	...	1.0	20	....	24,000	21,000	13,000	8,000

Temperature, 22°–24°.

EXPERIMENT 6.

STARTED FEBRUARY 20, 1906.

Bottle No.	Water (c.c.)	$CuSO_4$ Sol. (c.c.)	K_2SO_4 Sol. (c.c.)	Culture	Concent. of Salt (N/1,000,000)	Copper (Parts per Mill.)	Counts	
							Initial	24 Hrs.
1	98	1	..	2,006	20	0.63	44,000	460
2	98	..	1	"	20	....	50,000	49,000
3	99	1	..	A	20	0.63	69,000	600
4	98	..	1	"	20	....	90,000	44,000
5	98	1	..	B	20	0.63	45,000	400
6	97	..	1	"	20	....	48,000	26,000
7	98	1	..	C	20	0.63	61,000	600
8	98	..	1	"	20	....	67,000	25,000

Temperature, 21–23°.

EXPERIMENT 7.

STARTED MARCH 5, 1906.

Bottle No.	Water (c.c.)	Pepton (gm.)	Culture No.	$CuSO_4$ Sol. (c.c.)	Concentration of Salt (N/1,000,000)	Copper (Parts per Mill.)	Counts	
							Initial	24 Hrs.
1	100	0	2,006	0.5	10	0.31	179,000	1,040
2	100	0	"	2.0	40	1.24	189,000	3
3	101	0	A	0.5	10	0.31	2,400	3
4	100	0	"	2.0	40	1.24	2,200	0
5	98	1	2,006	0.5	10	0.31	171,000	2,420,000
6	101	1	"	2.0	40	1.24	175,000	455,000
7	99	1	A	0.5	10	0.31	2,800	19,500
8	98	1	"	2.0	40	1.24	2,900	15,000

Temperature, 20°–24°.

DISCUSSION OF RESULTS.

Effect of organic matter in tap water.—In comparing the results of these experiments, the basis of comparison will be the number of surviving organisms in each case, expressed as percentage of the initial number. Other bases of comparison suggest themselves, but the one selected appears on the whole to be the most logical. In this way the effect of the copper upon those few comparatively resistant organisms, always found in experiments of this nature, is given predominant importance, while the effect upon that large percentage of organisms which is killed in all the experiments has comparatively little weight.

Such a comparison of the average results of Experiments 1 and 3, best water, with those of Experiment 2, tap water, gives the following figures:

Concentration of Copper (N/1,000,000)	Per Cent Surviving—24 Hrs.		Ratio (b÷a)
	Best Water (a)	Tap Water (b)	
10	0.009	5.800	644
20	0.004	0.280	70
40	0.004	0.053	13
60	0.000	0.000	

It is quite evident that the organic matter present in the tap water inhibits the toxic action of the copper, but the significant fact here is that there is no definite point in the series at which the addition of more copper to the tap water will produce a normal killing effect such as is produced in pure water. The effect of the organic matter

cannot be neutralized up to the concentration required for the complete elimination of the organisms. It would appear, therefore, that the apparent inhibition of the toxic effect of the copper is due to the formation of some non-toxic compound; that the formation of this compound is due to a reversible reaction which is complete only at the highest concentration of copper used; and that below this point the addition of increasing amounts of copper simply brings about a further reaction toward this non-toxic compound, producing a new condition of equilibrium according to the law of mass action.

Dextrose.—The effect of dextrose upon the copper sulphate seems to be of a quite different nature. The following figures are calculated from the results of Experiment 4:

Concentration of Copper ($N/1,000,000$)	Per Cent Surviving—24 Hrs.		Ratio ($a+b$)
	Without Dextrose (a)	With Dextrose (b)	
10	0.12	34.0	283.0
20	0.009	0.02	2.2

In the lower concentration dextrose neutralizes completely the toxicity of the copper, so that the effect is about equal to that in the control (Bottle 8). In the higher concentration the effect of the dextrose is almost nil. This result, taken in connection with the fact that the concentration of the dextrose is about $\frac{1}{18}$ molar, and that there are accordingly over 10,000 $C_6H_{12}O_6$ molecules to each copper ion in the one case and over 5,000 in the other, leads to the conclusion that the results obtained are not due to the formation of a non-toxic compound. A careful study of the electric conductivity of these solutions did not reveal any decrease in the normal conductivity of the copper due to the presence of dextrose. It may be presumed, however, that what has been called a "physiological compound" might be readily broken down under the influence of the electric current.

The following assumption seems to be in agreement with all the facts: The bacteria doubtless attract to themselves by a process of adsorption a certain amount of dextrose, and are thus surrounded by a solution of this sugar more concentrated than that existing in

the water. This tends to produce a difference in osmotic pressure between the free solution and this surrounding layer, and it may be that a certain definite concentration of the copper ions is necessary before this film of "osmotic tension" can be pierced. An analogous case is that of surface tension, which presents a certain resistance to the entrance of a non-wetted body.

Peptone.—Peptone was the third substance studied. The results of Experiment 7 show that a 1 per cent solution of peptone allows the typhoid organism to multiply even in the presence of rather strong copper-sulphate solution. Owing to the high electric conductivity of the rather impure peptone, the results of conductivity determinations are uncertain. There seems to be little doubt here, however, that an actual combination has taken place between the copper and the peptone. The addition of sufficient copper solution to the peptone solution to give a distinct color resulted in the formation of a colloidal-like solution, of a robin's-egg blue color.

These results have an important bearing upon many practical points in connection with the use of copper sulphate for the destruction of the typhoid organism. Results obtained in the laboratory in distilled water are not in the least indicative of what may be expected under field conditions. Neither are actual field results on one water reliable criteria for the undertaking of similar work upon another. The impracticability of the internal use of copper sulphate in the treatment of typhoid fever is also suggested by the results obtained with peptone.

Selection of a resistant strain.—Cultures A, B, and C were taken from Bottle 2 in Experiment 5, and had lived for 48 hours in a solution of copper sulphate containing 0.63 parts of copper per million. They were used in Experiments 5 and 6 in parallel with the original strain in order to determine whether they possessed any increased resistance to copper. The results are all negative, indicating that these strains are not more resistant than is the parent strain.

The time factor in the germicidal effect.—In Experiments 1, 3, and 4, where determinations of the numbers of organisms were made at various intervals during the experiment, it is seen that even in the more dilute solution used there is a very rapid falling-off in numbers during the earlier part of the test. In Experiment 1, for instance,

the reduction in 5 minutes was 67 per cent in a concentration of 0.38 parts of copper per million, but all the organisms had not been killed in 24 hours. Such results indicate an extreme range of resistance to copper among these organisms. The 42 organisms which survived the treatment for 24 hours lived 288 times as long as the 250,000 which died in less than 5 minutes.

Effect of concentration.—This great variability is also shown in a study of the effect of various concentrations.

The average percentage survival after 24 hours in all experiments with 0.38 parts of copper per million was 0.19; with 0.76 parts, 0.07; with 1.26 parts, 0.04. Notwithstanding the fact that 99.8 per cent are killed by a concentration of 0.38 parts, there are still 0.04 per cent which can withstand the action of a copper solution four times as concentrated.

This tremendous variability is of vital importance, not only in questions of sterilization of water by heat, freezing, or chemicals, in all of which cases such a variability has been shown to exist, but in the far more important questions of the self-purification of streams and the longevity of the typhoid bacillus in the water. Most cases of typhoid fever, contracted from drinking-water, have undoubtedly come from these few resistant forms rather than from those which are known to have perished in the natural stream. A removal of 99.99 per cent of the typhoid organisms may sound like security but actually means high typhoid rates. The importance of this residual hundredth of a per cent cannot be overestimated, owing to the very fact that it is so resistant.

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A NEW SOLUTION FOR THE PRESUMPTIVE TEST FOR *BACILLUS COLI*.*

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MACCONKEY[1,2,3] has pointed out that the intestinal bacteria grow well on an agar medium containing 0.5 per cent of sodium taurocholate, while the common bacteria are to a great extent excluded, and he has recommended that this medium be used to distinguish between *B. coli* and *B. typhi abdominalis* and also as a test for fecal contamination in water. Jordan, Russell, and Zeit,[4] in experimenting with this medium for testing water, did not obtain very favorable results in that the medium failed to eliminate all of the water forms. Robin[5] has used MacConkey's agar to advantage in tests of filters; but while the results were preferable to those obtained on the regular agar, it could not be concluded that all the bacteria found, or a definite percentage of them, were always of fecal origin.

In order to determine the germicidal action of the bile salts and their constituents, the author has used a standard agar medium with an addition of varying amounts of these salts, and noted the effect produced. The following are the results obtained with the bile salts, and their constituents upon the agar growth at 37° C. of bacteria from a slightly contaminated water containing two *B. coli* per c.c.

TABLE 1.

	Plain Agar	Agar +0.05%	Agar+0.5%	Agar+2.5%
Sodium taurocholate	20	14	4	2
" glycocholate	20	14	2	2
Taurocholic acid	20	10	2	0
Glycocholic acid	20	12	5	0
Cholic acid	20	14	2	0
Taurin	20	20	19	20
Glycin	20	19	11	5

This table shows that the bile acids reduced the number of bacteria even when only 0.05 per cent was taken, and that when 0.5 per cent was employed, taurocholic, gylcocholic, and cholic acids, sodium taurocholate, sodium glycocholate, and glycin greatly reduce the bacteria present, some of those remaining giving the tests for

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B. coli. When 2.5 per cent of the salts were used the bacteria were killed by the bile acids and considerably reduced by glycin, while the results with the bile salts remained about the same.

It is evident that the bile salts and especially their acids exert a strong restraining action on the common bacteria which grow at blood heat, and that except for glycin (which mildly restrains much as a sugar retards bacterial action), the actual bactericidal effect lies in the cholic acid radical of these salts.

It is evident, therefore, that either of the bile salts or a mixture of both salts may be employed, thus greatly reducing the cost and ease of preparation of what would otherwise be a medium impracticable for use in laboratories where much water work is carried on. In fact it allows of the use of plain bile as a liquid restraining medium.

In order to determine whether or not this restraining action is selective, a series of waters and solutions were made containing varying numbers of intestinal bacteria. The following table gives the results obtained:

TABLE 2.

COMPARISON OF RESULTS FROM VARIOUS BILE AGAR MEDIA.

	Bacteria per c.c.			
	Uncontaminated Shallow Dug Well	Contaminated Pond Water	Suspension of Feces Previously Grown at 37° C.	Fresh Suspension Containing *B. coli.*
Gelatin at 20° C.	920	2700	350,000	900,000
Agar at 38° C.	25	170	450,000	900,000
Bile agar (fresh ox bile and agar)	0	16	60,000	900,000
Bile agar (with bile diluted 1–1)	14	43	300,000	900,000
Lactose bile agar (with bile diluted 1–1)	0	25	250,000	675,000
Litmus lactose bile agar (with bile diluted 1–1)	0	17	250,000	600,000

The media used in the experiments shown in the foregoing table were made from fresh ox bile instead of the usual meat infusion with peptone. The best results were obtained when the bile was not diluted.

The figures show that a strong restraining action is exerted against the growth of the common bacterial flora and to little or no extent against certain of the fecal bacteria.

The test for fecal bacteria is an important matter, especially in water analysis. The method generally employed at the present time for routine work was devised by Dr. Theobald Smith,[6,7,8] and con-

sists of obtaining the percentage of gas formed by bacterial growth in the closed arm of an inverted tube containing a solution of meat extract, peptone and lactose or dextrose. One-tenth, 1, and 10 c.c. of water are added to the sterilized media and allowed to stand 48 hours in an incubator at 37.5° C. At the end of this time the percentage of gas formed and the amount absorbed by caustic is obtained.

Typical growths of *B. coli* give from 25 to 70 per cent gas, of which from 25 to 40 per cent is carbonic acid and is absorbed by caustic. This so-called "presumptive test" is used extensively in most water laboratories, and in those of the New York City Department of Water Supply, Gas, and Electricity there are tested annually in this manner about 7,000 samples of water. The difficulty with this test lies in the fact that when badly contaminated water is taken as, for instance, a suspension of feces or a strong sewage, the test often utterly fails, due to an overgrowth of some germ other than *B. coli.*

In such cases *B. coli* is inhibited in its growth by the products of metabolism of certain other species, usually streptococci. This inhibition has been noticed by Prescott and Baker,[9] Irons,[10] and others, and has been a constant source of annoyance to the author in work on water and sewage. The use of sodium taurocholate as suggested by MacConkey to prevent the growth of these inhibiting bacteria is a step in the right direction, but the salt is very expensive and difficult to obtain, and the amount suggested (0.5 per cent), while effective in solid media, is, in the opinion of the author, too small for liquid media.

Inasmuch as the foregoing experiments showed an equal effectiveness of the more abundant sodium glycocholate there appeared to be no reason why a mixture of the two bile salts (Platner's crystallized bile) could not be used. This mixture is easy to obtain and cheap enough to use with liquid media in larger amounts than those employed by MacConkey. It is made as follows:

Concentrate ox bile to one-fourth its bulk; mix with animal charcoal in a mortar to a thick paste and evaporate to complete dryness over a water bath.

To the dry charcoal bile mixture add five volumes of absolute alcohol. Shake from time to time, and in about half an hour filter off the alcohol. Concentrate the filtrate by boiling; and when cold add ether in large excess and the crystallized sodium salts of taurocholic and glycocholic acid will be precipitated. Allow the precipitate to stand over night in a tightly covered glass jar and decant off the mixture of alcohol and ether.

Dry the precipitated salts first on the water bath and then in an oven kept at 100° C. Powder in a mortar and keep in a tightly stoppered, wide mouth glass bottle. Purify if necessary, by redissolving in a small quantity of warm alcohol and again precipitating with a large excess of ether.

The author's experiments with bile agar suggested the use of bile itself with 1 per cent of lactose as a liquid to replace Smith solution, thus making a much more effective medium which would be cheaper and easier to prepare than the latter solution. In the following experiments the results with the new bile lactose medium is compared with plain Smith solution, Smith solution containing sodium taurocholate, and the same solution containing sodium glycocholate.

It is evident that the presence of either or both of the bile salts favors the growth of *B. coli* in Smith solution by inhibiting the growth of other bacteria. A proper medium, may, therefore, be made by adding to Smith solution 9 per cent of Platner's crystallized bile

TABLE 3.

Test on Sewage	Total Gas	After Absorption	% Carbonic Acid	Test for *B. coli*
Smith solution—				
0.1 c.c.	12	—	—	0
1.0	12	—	—	0
10.0	17	—	—	0
Smith and taurocholate—				
0.1 c.c.	52	33	37	+
1.0	43	27	37	+
10.0	65	40	38	+
Smith and glycocholate—				
0.1 c.c.	33	23	30	+
1.0	26	18	31	+
10.0	47	31	34	+
Bile lactose—				
0.1 c.c.	25	18	28	+
1.0	35	25	29	+
10.0	27	19	30	+

Test on Solution of Horse Feces	Total Gas	After Absorption	% Carbonic Acid	Test for *B. coli*
Smith solution—				
0.1 c.c.	0	0	0	0
1.0	0	0	0	0
10.0	0	0	0	0
Smith and taurocholate—				
0.1 c.c.	0	0	0	0
1.0	25	18	28	+
10.0	34	23	32	+
Smith and glycocholate—				
0.1 c.c.	33	23	30	+
1.0	18	—	—	0
10.0	26	18	31	+
Bile lactose—				
0.1 c.c.	25	17	32	+
1.0	40	28	30	+
10.0	67	41	39	+

prepared as previously described, or by using the author's bile lactose medium.

This latter medium is to be preferred when fresh bile can be obtained, as it appears to be somewhat more effective and is cheaper and easier to prepare. Several brands of inspissated bile were tried but they were all very acid and even when neutralized were not effective. The bile lactose medium is prepared in the following manner:

Fresh ox bile from the slaughter house is poured into flasks and sterilized 20 minutes under 15 pounds pressure. When ready for use the bile is filtered and 1 per cent lactose is added. It is then poured into Smith tubes and again sterilized. If the bile is fresh the acidity will be from zero to 5 by Fuller's scale, and within these limits there is no interference with the results.

A large number of tests for *B. coli* were carried on to determine the relative efficiency of bile media as compared with Smith's solution, and also to show the selective action of the bile salts. Some of the most conclusive results are given in Tables 3 and 4.

TABLE 4.

COMPARISON OF RESULTS OBTAINED IN THE TEST FOR *B. Coli* WITH SMITH SOLUTION AND WITH LACTOSE BILE.

	Smith Solution			Bile Lactose		
	0.1 c.c.	1 c.c.	10 c.c.	0.1 c.c.	1 c.c.	10 c.c.
Solution No. 1 containing pure culture *B. Coli*—						
Total gas	36	37	40	32	46	45
After absorption	22	22	25	20	28	27
Per cent carbonic acid	38	40	28	37	39	40
Test for *B. Coli*	+	+	+	+	+	+
Solution No. 2 containing pure culture *B. Coli*—						
Total gas	30	27	60	49	65	53
After absorption	20	19	45	33	45	26
Per cent carbonic acid	33	30	25	33	26	34
Test for *B. Coli*	+	+	+	+	+	+
Pond water, slightly contaminated—						
Total gas	0	0	36	0	0	33
After absorption	0	0	24	0	0	21
Per cent carbonic acid	0	0	33	0	0	36
Test for *B. Coli*	0	0	+	0	0	+
Pond water, contaminated—						
Total gas	0	10	72	0	41	52
After absorption	0	—	40	0	23	29
Per cent carbonic acid	0	—	44	0	44	45
Test for *B. Coli*	0	0	+	0	+	+
New York Bay water, contaminated, No. 1—						
Total gas	25	17	10	43	35	57
After absorption	18	—	—	22	19	32
Per cent carbonic acid	28	—	—	49	46	44
Test for *B. Coli*	+	0	0	+	+	+
New York Bay water, contaminated, No. 2—						
Total gas	26	10	13	29	35	44
After absorption	20	—	—	16	22	25
Per cent carbonic acid	23	—	—	45	37	43
Test for *B. Coli*	0	0	0	+	+	+

TABLE 4.—*Continued.*

	Smith Solution			Bile Lactose		
	0.1 c.c.	1 c.c.	10 c.c.	0.1 c.c.	1 c.c.	10 c.c.
Rahway River, N. J., just below sewer outlet—						
Total gas	0	13	11	50	29	30
After absorption	—	—	—	28	17	18
Per cent carbonic acid	—	—	—	44	41	40
Test for *B. Coli*	0	0	0	+	+	+
Bodine Creek, S. I., badly contaminated—						
Total gas	17	37	36	53	32	65
After absorption	—	25	25	30	23	42
Per cent carbonic acid	—	37	27	40	28	38
Test for *B. Coli*	0	+	+	+	+	+
Kill von Kull, badly contaminated—						
Total gas	10	21	8	35	32	48
After absorption	—	—	—	22	23	33
Per cent carbonic acid	—	—	—	31	28	31
Test for *B. Coli*	0	0	0	+	+	+
Hudson River at mouth, badly contaminated—						
Total gas	15	5	8	32	33	25
After absorption	—	—	—	18	17	16
Per cent carbonic acid	—	—	—	44	51	36
Test for *B. Coli*	0	0	0	+	+	+
Hudson River at Hoboken, badly contaminated—						
Total gas	12	10	13	27	20	42
After absorption	—	—	—	17	—	27
Per cent carbonic acid	—	—	—	37	—	36
Test for *B. Coli*	0	0	0	+	0	+
East River near B'klyn Bridge, badly contaminated—						
Total gas	10	41	13	51	34	40
After absorption	—	30	—	26	25	26
Per cent carbonic acid	—	26	—	49	26	30
Test for *B. Coli*	0	+	0	+	+	+
East River below Blackwell's Island, contaminated—						
Total gas	25	19	22	25	27	35
After absorption	17	—	—	17	17	20
Per cent carbonic acid	32	—	—	32	37	43
Test for *B. Coli*	+	0	0	+	+	+
Harlem River near mouth, contaminated—						
Total gas	20	12	40	27	26	25
After absorption	—	—	27	19	19	16
Per cent carbonic acid	—	—	33	30	27	36
Test for *B. Coli*	0	0	+	+	+	+
Bronx River, contaminated—						
Total gas	33	22	23	33	50	33
After absorption	23	—	—	23	30	24
Per cent carbonic acid	30	—	—	30	40	27
Test for *B. Coli*	+	0	0	+	+	+
Gowanus Canal, at head, badly contaminated—						
Total gas	15	13	17	27	26	33
After absorption	—	—	—	17	15	20
Per cent carbonic acid	—	—	—	27	42	40
Test for *B. Coli*	0	0	0	+	+	+
Gowanus Canal at Hamilton Avenue bridge, badly contaminated—						
Total gas	12	17	18	45	47	52
After absorption	—	—	—	30	30	27
Per cent carbonic acid	—	—	—	33	33	48
Test for *B. Coli*	0	0	0	+	+	+
Sewage effluent after rough filtration, Bedford, N. Y.—						
Total gas	12	12	12	23	35	25
After absorption	—	—	—	—	25	18
Per cent carbonic acid	—	—	—	—	29	28
Test for *B. Coli*	0	0	0	0	+	+

TABLE 4.—*Continued.*

	Smith Solution			Bile Lactose		
	0.1 c.c.	1 c.c.	10 c.c.	0.1 c.c.	1 c.c.	10 c.c.
Sewage, outlet 65th St. sewer Brooklyn, N. Y.—						
Total gas	7	26	20	27	39	20
After absorption	—	17	—	16	23	—
Per cent carbonic acid	—	35	—	41	41	—
Test for *B. Coli*	0	+	0	+	+	0
Water suspension No. 1, fresh horse feces—						
Total gas	0	0	0	23	46	60
After absorption	0	0	0	—	32	30
Per cent carbonic acid	0	0	0	—	45	25
Test for *B. Coli*	0	0	0	0	+	+
Water solution No. 2, fresh horse feces—						
Total gas	0	0	0	25	40	67
After absorption	0	0	0	16	28	41
Per cent carbonic acid	0	0	0	36	30	39
Test for *B. Coli* d	0	0	0	+	+	+
Human feces No. 1 kept in water two weeks at 37° C.—						
Total gas	5	15	42	0	41	37
After absorption	—	—	28	0	25	37
Per cent carbonic acid	—	—	33	0	39	35
Test for *B. Coli*	0	0	+	0	+	+
Human feces No. 2 kept in water two weeks at 37° C.—						
Total gas	12	8	10	46	50	61
After absorption	—	—	—	28	29	34
Per cent carbonic acid	—	—	—	39	42	44
Test for *B. Coli*	0	0	0	+	+	+
Water suspension No. 1, fresh human feces—						
Total gas	18	18	7	44	50	61
After absorption	—	—	—	25	35	32
Per cent carbonic acid	—	—	—	43	40	47
Test for *B. Coli*	0	0	0	+	+	+
Water suspension No. 2, fresh human feces—						
Total gas	5	18	23	26	27	32
After absorption	—	—	—	19	20	22
Per cent carbonic acid	—	—	—	27	26	31
Test for *B. Coli*	0	0	0	+	+	+

In the investigation of the relative efficiency of the Smith and lactose bile solutions, 275 badly contaminated waters were examined, of which 65 per cent gave improper results with the use of the Smith solution while only 10 per cent of overgrowths were found when lactose bile was used. The total amount of gas produced and the percentage of absorption is generally greater when lactose bile is used. The gas usually forms somewhat more slowly in the lactose bile, and while the second day's results are preferable to those obtained by the Smith solution, still better tests are obtained by incubating three days.

CONCLUSIONS.

The bile salts, and especially their acids, exert a strong restraining action on most species of bacteria which grow at blood heat.

This restraining action is selective. It favors the increase of *B. coli*, retards the growth of certain streptococci, and actually kills off the majority of species which grow at 37° C.

The effect is due to the cholic acid radical and is, therefore, common to both of the bile salts.

Advantage may be taken of the selective action of the bile salts in the determination of *B. coli* in water by planting various amounts of the water to be tested in bile lactose solution. The results are much more accurate than those obtained by the methods at present employed.

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B. COLI IN MARKET OYSTERS.*

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SINCE the considerable mass of work on the subject of typhoid fever and oyster infection has been recently reviewed by G. W. Fuller,[1] there is no need of summarizing it in detail here. One aspect of the subject, which is of importance from the public-health standpoint, seems, however, to have received little attention. Although oysters have been taken from various beds and examined for sewage pollution, I believe no extended examination has been made in this country of oysters obtained from city markets. In England Herdman and Boyce[2] examined oysters from various shops, and in a very large proportion of cases *B. coli* was isolated.

It is a matter of considerable importance to know how large a proportion of commercial shell oysters on sale in a given locality are polluted. Knowledge of this sort will aid the health authorities of a city in detecting possibilities of danger and in drying up the source of infection. With this object in view, examination has been made of shell oysters from a number of the principal Chicago markets.

It has been well established that the presence of *B. coli* in oysters indicates sewage pollution. C. A. Fuller[3] has studied the relation between oysters and sewage in Narragansett Bay, and has shown that bacterial analyses of oysters correspond closely with analyses of river water above the beds and with the opportunities for contamination as determined by inspection.

Considering *B. coli* as an index of pollution, the examination was carried on by the following method.

Each oyster was opened with sterile instruments, carefully removed from its shell, and after being rinsed in sterile water was placed in a Petri dish. The oyster was then finely minced with sterile scissors and mixed with 5 c.c. of sterile water. A dextrose fermentation tube was inoculated with 1 c.c. of the fluid from the minced oyster. If no

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[1] *Jour. Franklin Inst.*, August, 1905, p. 81. [2] *Thompson-Yates Laboratories Rep.*, 1899, 2, p. 43.

[3] Appendix to 1904 *Rep. U. S. Commissioner of Fisheries*, pp. 189–238; *Science*, 1903, 17, p. 371.

gas formed in the tube after 48 hours at 37° C.,the test was considered negative. However, if gas did form, a litmus lactose agar plate was made as soon as possible, and after 24 hours agar tubes were inoculated from the red colonies. The pure cultures were then studied. Every fermentation tube not showing gas was examined at the end of 48 hours for growth in the closed arm, and a note made of the existing condition.

One c.c. of fluid from the oyster was thought to be sufficient to inoculate the dextrose tube to show any gas-forming organisms present in the oyster. In order to be more certain of that point, several fermentation tubes were inoculated from one oyster, and the tubes all showed the same results. The same process was repeated on several occasions.

As far as could be discovered, the majority of Chicago market oysters come from Baltimore and New York; some come from Connecticut. The larger part of the supply is from Baltimore.

Oysters from the following markets were examined:

Market No. I. Large wholesale and retail market. Supplies oysters to many of the smaller markets in Chicago. "Oysters from Baltimore."

Market No. II. Wholesale and retail market. "Oysters from New York."

Market No. III. Wholesale and retail market. "Oysters from Baltimore."

Market No. IV. Large retail market. "Oysters from Connecticut."

Market No. V. Retail market. "Oysters from Baltimore."

Market No. VI. Retail market. "Oysters from New York."

Market No. VII. Retail market. "Oysters obtained from wholesale market No. I."

Market No. VIII. Retail market. "Oysters from wholesale market No. I."

Market No. IX. Retail market. "Oysters from New York."

At numerous other markets visited it was found that the oysters were obtained from some of the wholesale markets above mentioned. The source of the oysters has been recorded just as the information was received at the markets.

The table following shows the results of the examination.

As the figures in the table show, the oysters examined from market No. I were free from any indication of sewage pollution. Only four dextrose tubes from 63 oysters showed gas; from those tubes only proteus forms were isolated.

From market No. II 24 oysters were examined. Two dextrose tubes showed gas. From one, an organism belonging to the colon

TABULATED RESULTS OF THE EXAMINATION.

Market	Number of Oysters Examined	Fermentation Tube. Growth in Closed Arm after 48 Hours But no Gas	Oysters Showing *B. Coli* or Coli-like Forms	Oysters Showing Proteus Forms	Other Gas-Forming Organisms
No. I, March 7	25	2	0	1	0
" " 14	26	1	0	3	0
" " 23	12	1	0	0	0
No. II, " 21	12	4	1	0	0
" " 26	12	3	0	1	0
No. III, " 21	14	7	1	0	0
" " 26	12	7	0	1	0
No. IV, " 6	26	14	1	0	0
" " 13	32	2	0	0	0
No. V, February 27	12	2	7	1	*B. cloacae* 2
" March 20	26	5	0	0	0
No. VI, February 23	7	3	0	1	*B. cloacae* 1
" " 26	10	5	0	0	0
" March 5	22	11	0	9	0
No. VII February 26	17	5	1	10	*B. cloacae* 1
No. VIII, March 19	15	14	0	1	0
No. IX, March 24	14	0	0	0	0

group was isolated, from the other, a proteus form. From the finding of one coli-like organism in 24 oysters it could not be said that the oysters were seriously polluted. However, it seems to show the possibility of pollution and indicates that the oyster beds were probably located in a more or less contaminated water.

From market No. III 26 oysters were examined. Two dextrose tubes showed gas. One proteus and one organism of the colon group were isolated. The same may be said of that supply as was said about the oysters from market No. II.

The same applies to the oysters from market No. IV, of which 58 were examined; one coli-like organism being isolated.

Regarding the oysters from market No. V, there is no doubt that the first lot, collected February 27, was badly sewage-polluted. From seven of the 12 oysters examined colon forms were isolated. The oysters did not appear to have been fattened and seemed perfectly fresh. On attempting, some two weeks later, to obtain more oysters at the market, it was found that the dealer no longer kept shell oysters for sale. He explained that the oysters "went bad" and opened before he could sell them. The market supplied oysters, however, if they were ordered. On examination of 26 oysters thus obtained, none of the tubes showed gas. It was impossible to find out just where the oysters came from, except that both lots came from Baltimore.

From market No. VI 39 oysters were examined. No colon bacilli were found. The supply was evidently free from pollution.

The oysters from markets Nos. VIII and IX showed no organisms of the colon group.

The oysters from market No. VII, although perhaps not showing indications of serious pollution, seemd to be in a state of decomposition which would render them unsuitable for consumption. From 17 oysters, one organism of the colon group and 10 proteus forms were isolated. The oysters were no doubt old, as indicated by the fact that their shells were slightly open. The presence of proteus forms probably shows that the oysters were undergoing decomposition, which view is further borne out by the putrid odor which accompanied them.

From the results of the examination it seems likely that growth in the closed arm of the fermentation tube, without gas formation, indicates the aging of the oysters. The presence of proteus forms probably means old oysters.

SUMMARY.

1. Eleven organisms belonging to the colon group were isolated from the 294 oysters examined.

2. Colon bacilli were found in oysters from five markets out of a total of nine.

3. The presence of *B. coli* in so large a proportion of oysters from market No. V seems to show that the oysters had been in contact with sewage. A second lot of oysters from the same market, but from another source, showed no evidence of pollution.

4. Colon bacilli in so small a proportion of oysters in Chicago markets would hardly indicate a widespread pollution, but a more extended examination might show different results.

5. The colon test seems to afford a valuable means for determining the purity of a city oyster supply.

It is not intended to present these facts as a complete study of the oyster supply of Chicago, but simply to show the condition of oysters from several of the most important markets in order to illustrate the value of such a bacterial examination.

STUDIES ON SIMPLE AND DIFFERENTIAL METHODS OF STAINING ENCAPSULATED PNEUMOCOCCI IN SMEAR AND SECTION.*

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IN the course of some experimental studies on pneumococcus infection, the technic of previous observers recommended for the staining of the encapsulated organisms was tested, and various new methods were devised in the hope of securing reliable procedures by which pneumococci may be differentially stained in cover-glass preparations and in sections of tissue.

My experience with these methods, old and new, it is the purpose of this paper to record.

PREVIOUS METHODS.

Simple staining.—In the simple† routine staining with aqueous-gentian-violet or carbol fuchsin, smears of pneumococcus exudates may show the organisms encapsulated; but this is extremely uncertain. Similarly, under favorable conditions many of the older special methods‡ devised for capsule staining often give excellent preparations, but the results vary, and are therefore unreliable when compared with those obtained with the simple, perfected technic used by recent observers. The most reliable and practical of all these methods in my experience are based wholly or in part on principles first adopted by Guarnieri.

In 1888 Guarnieri[5] made determinations of the solubility of the pneumococcus capsule in acid, alkaline, and neutral salt solutions; and finally he obtained the proteid reaction with Millon's reagent, thus indicating an albuminous composition. On these determinations Guarnieri devised a new method of staining the encapsulated organisms in exudates: smears fixed in the flame

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†Pane.[10]

‡Friedländer,[3] Ribbert,[13] Roux,[14] Muir,[11] MacConkey,[9] Gordon,[4] Kolle and Wasserman.[8]

were stained with analin-gentian-violet, then washed and differentiated in 2 per cent aqueous sodium chloride, rewashed very quickly in water, dried, and mounted in balsam. Similar methods have since been recommended. Thus, Welch[16] adopted Guarnieri's method of staining, but mounted the specimen in the salt solution, and suggested a preliminary treatment with glacial acetic acid on the ground that the capsule was composed of mucin.*

Buerger[2] also adopted Guarnieri's method, but recommended a preliminary fixation of the capsule, first in a solution of chromic acid and bichloride of mercury, then in an alcoholic solution of iodine (U. S. P.). With Gram's method of staining this fixation is essential, but in the simple procedures the advantage of it is less apparent, for practically the same results are secured with Guarnieri's less complicated method.

Hiss,[6] however, by substituting the ordinary aqueous-gentian-violet stain for the unstable anilin-gentian-violet, and by using 0.25 per cent potassium carbonate solution, which to some extent clears the field, simplified and improved materially the technic of capsule staining. Finally, by using a 20 per cent copper sulphate wash instead of the potassium carbonate he found that the specimen could be dried and mounted in balsam.

Formerly capsules were found only in the exudates of infected animals, but now they are readily demonstrated in organisms growing in artificial media. Boni found that when cultures of pneumococci are smeared in egg albumin, the capsules are easily stained. Hiss secured similar results with blood serum, and by means of his more reliable methods of staining was able to determine more fully the importance of utilizing this principle in the morphological study of the pneumococcus.

By virtue of these modern procedures it is now a comparatively simple matter to demonstrate capsules on these organisms. It is no longer a question of how encapsulated pneumococci may be stained, but of how they may be most simply and reliably stained.

*Welch does not state the reasons for this belief; it is therefore difficult to refute the positive, though incomplete, observations of Guarnieri. Mucin is a glycoproteid and reacts to Millon's reagent, but the solubilities differ, and capsules are more constantly noted in albuminous media than in the presence of mucin. In fact, the mucous secretions of the mouth are not a particularly favorable environment for the demonstration of capsules, whereas capsules are readily obtained in simple broth, if only the albumin or even the peptones be sufficiently increased.

The methods of Guarnieri, of Welch, and of Buerger, reliable, though in one way or another complicated, all give temporary mounts, and are thus unsatisfactory; particularly when similar or better results may be secured by simpler procedures giving permanent preparations in balsam, which may be kept for future reference, as with the copper sulphate method of Hiss.[6]

Differential staining.—These simple methods of staining encapsulated pneumococci, however, do not always suffice to differentiate the pneumococcus from other capsule-forming bacteria.* To complete this differentiation further steps are necessary. In pneumonic exudates, pneumococci, streptococci, and pneumobacilli (Friedländer) are frequently associated together. The streptococci, except possibly under especially favorable conditions, rarely show capsules. The pneumobacilli not only are encapsulated, but the short and degenerating forms are practically indistinguishable from the pneumococci. The pneumobacillus, however, decolorizes by the Gram stain; but this does not show capsules, and when these contaminated exudates are examined for the morphological determination of the presence of these species, both the capsule and the Gram stains are required, and even then the observations may not be accurately correlated. Cultural characters are more precise and final, but require time; and, furthermore, the streptococci and pneumobacilli, when present, usually predominate; they also grow so rapidly, and the pneumococci are frequently in such small numbers, that, unless exceptional precautions are taken, the pneumococci may not be found.

For these reasons efforts have been made to obtain a reliable method which would demonstrate the capsules and the Gram differential in the same preparation. This result has been noted in specimens overexposed, or exposed with heat to the anilin stain and the iodine solutions; but this is so exceptional that special methods have been devised.

For this purpose Smith[15] fixes the smears of fresh sputum from pneumonia patients in the usual way by heat. The films are then

*The morphological differences in the capsules of the pneumococci, as compared with other encapsulated organisms resembling the pneumococcus, which Buerger obtained with his simple stain, and upon which he lays so much stress, depend chiefly upon the varying stages of development or degeneration and solution of the capsule, and upon the degree of decolorization. They are in no sense differential.

steamed in anilin-gentian-violet and in the iodine solution. After the usual alcohol decolorization and a few seconds' exposure to a mixture of alcohol, 4 parts, ether, 6 parts, the smear is counterstained, first in aqueous eosin, then in Loeffler's methylene blue. This is followed by slight decolorization in 95 per cent alcohol, dehydration in absolute alcohol, xylol, and balsam.

Buerger[2] fixes the smears of encapsulated pneumococci in Müller's fluid saturated with bichloride, for one-half minute,* and washes in water. After one minute's exposure to an alcoholic solution of iodine (U. S. P.), and washing in alcohol, the smear is stained in the usual way by the Gram method—anilin-gentian-violet, Lugol's solution, alcohol decolorization—and washed in water. After counterstaining in aqueous fuchsin, the preparations are washed and examined in 2 per cent salt solution.

With these methods of Smith and of Buerger the bacterial cells, under favorable conditions, may be stained by the Gram stain and demonstrated with capsules. The procedures, however, are complicated, not as accurate and reliable as the simple capsule stains, or give temporary mounts; and none of the methods thus far considered are applicable to the demonstration of encapsulated organisms in sections of diseased tissues.

THE NEW METHODS.

In the attempt to secure methods by which the differential staining of encapsulated organisms in tissues as well as films could be easily accomplished with a reasonable degree of certainty, a great variety of procedures were tried without success before satisfactory results were finally obtained. Suffice it to note that all the efforts to keep the capsules from dissolving in the course of hardening, imbedding, and staining, by the addition of various chemicals to the solutions, so successfully adopted in the simple methods of Guarnieri and of Hiss for films, failed to give reliable results. Exceptionally, a few encapsulated organisms were stained in sections treated in this fashion, but the result of these procedures proved to be wholly beyond control, and was thus of no practical value. It was therefore evident, if the Gram stain was to be used, that the solution of the problem depended primarily upon securing a per-

* In my experience with this method longer exposures are advisable; in fact, essential.

manent fixation or coagulation of the capsule. Obviously, success in securing permanent fixation depended upon the composition of the capsule. In the absence of data to the contrary, the results of Guarnieri's chemical studies, and the fact that capsule preservation or formation is largely determined by the presence of albumin, suggested with reasonable probability an albuminous composition. Attention was therefore directed to the study of the action of bichloride of mercury and formalin, which not only precipitate albumins, but enter into chemical combination with them.

Bichloride methods.—Bichloride of mercury was tested in both aqueous and alcoholic solutions. Alcoholic solutions proved more efficient. Dried smears, fixed in saturated bichloride-alcohol for a few minutes and washed in water, may be stained in aqueous-gentian-violet, and mounted in the usual way, or they may be decolorized by retreatment in the bichloride-alcohol for a few seconds before washing in water and drying. In these preparations the encapsulated cells are often very sharply demonstrated in a perfectly clear field. With the Gram differential procedures the capsules fixed in bichloride-alcohol rarely* withstand the osmosis required to bring the specimen to a balsam mount, and as applied to the study of tissues in section it was a complete failure.

Bichloride-alcohol thus proved valuable in coagulating and partially fixing the capsules, and also in clearing the field; but, aside from this, it offered little practical advantage over the simple staining procedures of other observers.

Formalin methods.—The fixation obtained with strong solutions of formalin proved more stable. It was also found that, when desirable, formalin may be effectively used as a wash after simple staining with aqueous-gentian-violet, or 5 to 10 per cent may be added to the stain to shorten the procedure. Although useful and simple, these methods offer no special advantage over the definite fixation secured by the stronger (40 per cent) solutions of formalin,†

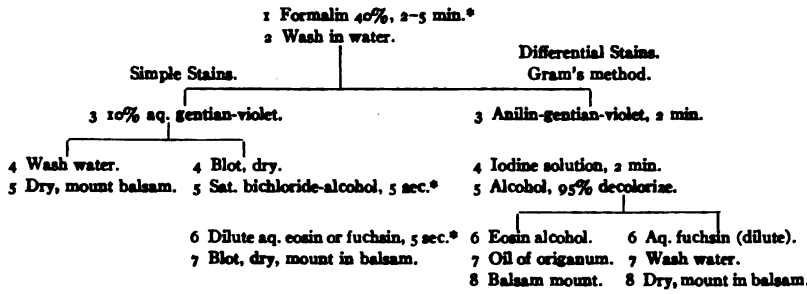
*Excellent balsam preparations for demonstration purposes were readily secured, but for routine work the results were uncertain. With other species of encapsulated bacteria this may prove more reliable, for in sections of tissues hardened in bichloride Wright and Mallory[17] demonstrated capsules on a Gram negative organism resembling the Friedländer bacillus.

†The addition of alkali to the formalin interfered seriously with the fixation, but the addition of acetic acid 1 to 2 per cent in some instances proved advantageous, especially in securing penetration of the tissues, and also it was thought, by counteracting the alkaline reaction of the body tissues.

after which a great variety of staining as procedures, differential well as simple, may be readily adopted according to the character of the material to be examined, and the purposes of the examination. The simple routine gentian-violet stain may be used, or this, after drying, may be decolorized for a few seconds in saturated bichloride-alcohol and counterstained, or the Gram differential method may be employed. Finally bits of diseased tissue may be hardened in 40 per cent formalin, imbedded in celloidin, and sections cut and stained by simple and differential methods to demonstrate the presence of encapsulated organisms.

Briefly tabulated, the technic for smear preparations is as follows:

TECHNIC FOR THE FIXING AND STAINING OF SMEARS.



THE TECHNIC FOR SMEARS OF SPUTUM.

The demonstration of encapsulated pneumococci in smears of exudates by these formalin methods is thus comparatively simple. The demonstration of encapsulated pneumococci in smears of sputum, however, has proved in my experience another problem. With fresh sputum containing exudate coughed up from the lung good results may often be secured, especially if the more purulent portions of the sputum are selected for examination. But more frequently the organisms fail to show good capsule development, and in the secretions from healthy persons the capsule is even less marked. Apparently saliva and mucus offer poor conditions for capsule formation, and, although the cells coming from the lung exudates may retain their capsules for some time, the organisms growing in the mouth secretions may lose their capsules. To demon-

*Thick smears require longer exposure than thin smears of material containing few cells and little detritus.

strate capsules on such cells, albumin* in the form of blood serum (Hiss) or egg albumin (Boni) must be added, and thoroughly mixed with the sputum before the smears are made. The mucus in which many of the organisms are imbedded protects them from the albumin, and they fail to show capsules. Often it is only the isolated or exposed cells which show capsules. In the fixation by formalin the mucus also protects the cells from the action of this reagent; longer exposure is therefore required, and after this the preparation should be thoroughly washed in water, or some of the formalin will be retained and precipitate the anilin stain when this is used.

The demonstration of capsules on the pneumococci in sputum thus depends primarily upon bringing the bacterial cells into an albuminous environment favorable to the preservation or development of capsules.† This accomplished, the staining procedure is purely a matter of choice. With the formalin preparations the Gram stain may be used and a differentiation for practical purposes at once secured, as on further study in culture, Gram-positive, encapsulated organisms of pneumococcus morphology practically always give the biological characters of the pneumococcus. Occasionally encapsulated forms of the *Micrococcus tetragenus* resemble atypical pneumococci, but usually there is little difficulty in differentiating these forms. The *Streptococcus mucosus*, in my experience, cannot be differentiated morphologically from the pneumococcus. Streptococci, in my experience, have rarely given definite capsules‡ with these formalin methods.

THE TECHNIC FOR SECTIONS OF TISSUES.

Although the fixation of the pneumococcus capsule in smears is simple, in tissues hardened for celloidin sections it is difficult and less certain. The chief difficulty lies in securing sufficient penetration. In the body fluids the formalin is diluted, and it combines with the albuminous material which is coagulated. This

*Blood serum seems to mix with sputum better than egg albumin, but as suggested by Professor F. C. Wood, egg albumin, if it is diluted and made isotonic with NaCl solution, will give practically as good results.

†With some pneumococci capsules are obtained with great difficulty. For complete discussion of the differentiation of the pneumococci the reader is referred to the work of Hiss, Borden, and Knapps.

‡Occasionally a faint, hazy periphery, suggesting a shrunken or degenerated, partially dissolved capsule, was noted.

protects many of the bacterial cells from the action of the formalin, and the capsules of these cells are not properly fixed, and are thus dissolved by subsequent treatment. These difficulties are often encountered in precise histological work, and are largely eliminated by injecting the tissue with the hardening fluid, or, when this is not feasible, by cutting the material into small bits or thin slices.

The lungs are easily injected through the trachea and are quickly hardened in the distended condition. The lesions may then be cut into small pieces and the fixation completed in fresh, strong formalin, the whole process taking from three to five hours. After alcohol dehydration the material is imbedded in celloidin and cut in the usual way. It is important to have thin sections* for microscopical examination; otherwise the encapsulated bacteria lying in the exudate among the cells cannot be easily distinguished. After alcohol dehydration these sections may be fixed on the slide by partially dissolving the celloidin with ether, or alcohol and ether, equal parts. A few seconds in alcohol will then harden the thin film of celloidin covering the section, and after washing in water the preparation is ready to be stained. A variety of staining procedures may be employed;† but the Gram method, by virtue of its differentiation, has proved the most useful. Anilin-gentian-violet stain for two to five minutes, iodine solution one to two minutes, alcohol decolorization, eosin-alcohol counterstain, eosin-oil of origanum to clear, and balsam mounting, are the several steps of the technic. This has rarely failed to give good results, but occasionally the pneumococci, after prolonged exposure, or after exposure to weak or impure formalin solutions, decolorize partially in the alcohol. This technical error, when it occurred, was rectified by using a 5 per cent bichloride-alcohol for the decolorization following the iodine solution, so that the material could be studied, though not so accurately as when the cells were properly fixed. By

*Thin sections are readily secured by painting the surface of the block with dilute celloidin-ether between each stroke of the knife.

†A combination of the Nicolle and Van Gieson methods of staining has given some excellent preparations. After staining in Loeffler alkaline methylene blue, the dye is fixed in the bacterial cells by 10 per cent aqueous tannic acid. This is followed by a partial decolorization in alcohol, counterstaining in van Gieson's strong fuchsin-picric acid solution (Freeborn, *Proc. N. Y. Path. Soc.*, 1893, p. 73), differentiation in picric acid alcohol, clearing in picric acid-oil of origanum, and mounting in balsam. The Gram stain may be substituted for the methylene blue and tannic acid stain, but it is apt to decolorize in the acid alcohol. The picric acid cellular stain is more easily studied than the eosin stain.

using strong bichloride-alcohol for decolorization, Gram-negative organisms retained the gentian-violet stain and were demonstrable in the sections.

The difficulties of staining encapsulated pneumococci in sections of diseased tissues are thus purely technical, similar to those met with in all precise histological work, and with due care easily eliminated. By using the Gram method of staining a practical differential procedure is available for the accurate determination of pneumococci in sections of diseased tissues. This is particularly valuable in the study of pneumonic lesions, where contaminations or secondary infections often occur, and cultural examination fails to reveal the true significance of the bacteria isolated, or the relationship of these organisms to the disease processes.

These methods of studying the encapsulated pneumococcus, uniform in principle, for the most part simple, and adaptable to varying conditions, have been of such value in my studies that I believe they may prove similarly useful to other workers in this field.

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AN APPARATUS FOR TESTING THE VALUE OF FUMIGATING AGENTS.*

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COINCIDENTLY with our increased knowledge of the part played by mosquitoes in the spread of malaria and yellow fever there has arisen a demand for a class of substances which shall be efficient in killing these insects.

Although the introduction of preventive measures for these diseases is a comparatively recent event, the number of substances—"Culicides"—proposed for this purpose is very great; in fact, there are approximately as many culicides as there are disinfectants for bacteria, although our knowledge of the latter is much the more complete.

There is this difference, however, between the two classes of substances above mentioned, namely that whereas the bacterial fumigation has been studied with great care and detail, with gradually perfected apparatus and methods, the mosquito fumigation is still in the rule-of-thumb state, and we have no very definite data based upon careful experimental procedures upon which to compare the relative efficiency of different culicides. This is due, to a considerable extent, to the fact that at present there is no method or apparatus which will allow such comparison.

The writer has had occasion to construct an apparatus for this purpose which has given satisfaction in actual use, and which has furnished a ready means of demonstrating the applicability of the various culicidal substances which from time to time have been proposed in this connection.

Before describing the apparatus in detail, it will be well to consider what one must know about a fumigant; confining ourselves to salient points, omitting details which are of lesser importance.

One must consider cost, availability (including continuous supply), killing (orculicidal power), effect upon furnishings (or in

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general upon material likely to be exposed to its action), and the possibility of leaving poisonous residues. Of these factors, the cost and supply are factors quite without the pale of any experimental data, aside from the question of cost in so far as it is affected by questions of relative efficiency, and need not concern us here.

The question of killing power, chemical changes, and action upon substances exposed to the action of the fumigant are points of the greatest importance.

It should be stated that there are two factors involved in the lethal action of fumigants upon mosquitoes: the first, a stupefying effect, which is, or was, overlooked for a time, and the actual death of the insect. Almost invariably stupefying precedes the death of the mosquito, although the latter may follow the former so quickly as to appear almost as a simultaneous phenomenon; hence it is necessary so to place insects upon which one decides to try the action of a fumigant that they may be freely exposed to the culicide, yet be available for examination at any period of the experiment.

The apparatus described below has been constucted with these points in view; in addition, provision is made for the introduction of samples of the various fabrics, paints, finishings, and, in general, any sort of material likely to be exposed to fumigation.

The latter is more important than would at first seem possible. For example, it has been found that certain paints, containing lead or similar metals, if poorly applied, or exposed to certain chemicals in the presence of excessive moisture, turn yellowish, or even dark-colored, due to the formation of sulphides. While this does not necessarily spoil the protective action of the pigment, it detracts greatly from the esthetic appearance and renders the fumigating squad liable for damages. This is a particularly important point in tropical countries, where the humidity is always excessive, and where much fumigation is necessary.

The principle involved in this apparatus is simple. The essential parts are a box having a content of 100 cubic feet, provided with a series of holes through which may be introduced cages, made of wire gauze (20 mesh) six inches long, one and one-half inches in diameter, closed at one end with wire netting of the same mesh as that forming the body of the cage, at the other end by a tapering

stopper of wood, which fits tightly into one of the holes in the side of the box mentioned above, in such a manner as to support the cage inside of the box with the long axis of the cage at right angles to the wall through which it projects, in which position it is maintained by the wooden stopper. It will be seen that the stopper does three things: it closes the open end of the netting cage, preventing the escape of mosquitoes or other insects which may be inclosed in the cage, stops the hole in the wall of the box through which the cage is introduced, and keeps the cage in position.

Mosquitoes placed in cages, which in turn are suspended from the wall of the box in the manner above described, are freely exposed to whatever may be present in the air within the box, because the gases or fumes pass freely through the wire gauze of which the cage is made; at the same time, the insects are imprisoned in the cages, and are available for close examination at any time merely by removing the stopper, which in turn removes the cage to which it is attached.

The hole occupied by the stopper and cage is closed by a similar stopper, when the cage is removed, thus preventing the escape of gases. Inasmuch as one may replace the cage by a blank stopper in a very few seconds, the loss of fumigating material is minimal.

Glass windows are provided in opposite sides of the box. These permit a limited examination of the contents of the box until the fumes become too dense. The relative amount of light coming through, observed at the windows of the box as fumigation progresses, furnishes a rough index of the volume, or rather density, of the fumes at any time. Hooks are provided in the interior of the box, upon which may be hung various fabrics; or one may place fabrics over the cages in such a manner as partially to shield the cages containing mosquitoes from the action of the fumigant.

In addition, a lamp (alcohol) with a long neck has been used to furnish heat or flame for any substances requiring the addition of heat aside from that produced by the combustion itself. The lamp is supported by a stopper which fits tightly in one of the holes in the bottom, through which the cages are introduced, and one may remove the lamp as one replaces the cages by removing the stopper, which withdraws the lamp, and substituting a blank stop-

per; one may thus introduce or remove the lamp at any time without interfering with the fumigation.

The removable cage is especially important for the study of the interval elapsing between stupefying and actual death of the mosquitoes. One places stupefied mosquitoes in the fresh air for a time, and in this way determines the interval necessary to produce the various sets of phenomena between the beginning of the experiment and the actual death of the mosquito.

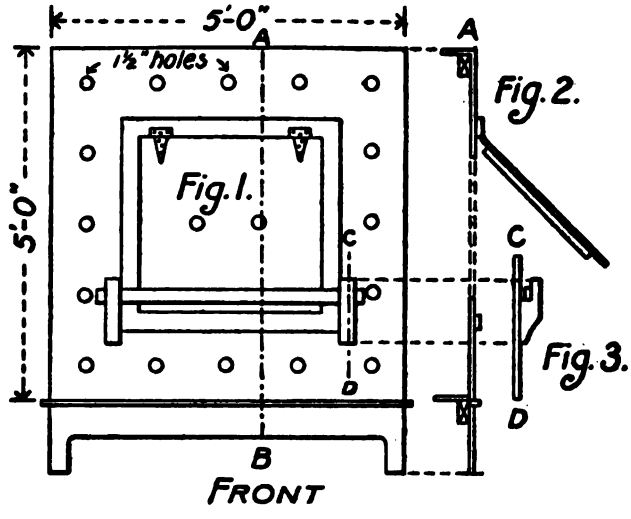


FIG. 1.—Front of fumigation apparatus.
FIG. 2.—Detail of door, showing overlapping surface.
FIG. 3.—Device for firmly closing door.

DETAILED DESCRIPTION OF THE FUMIGATING APPARATUS.

The box in which the experimental fumigations are carried on is made to contain exactly 100 cubic feet, inside measure. The dimensions are five feet wide, four feet long, and five feet high; this is approximately the average proportions of many rooms which are fumigated in Panama, and these dimensions have been chosen because it is believed that the ratio of height to length and width is not without influence in this work.

The material is pine, covered with three coats of white paint, to preserve the wood as well as possible from the action of moisture. The door and window in the back are made in such a way that

there is three inches' overlapping of the door and window upon the framing, to minimize the loss of fumigant. In actual practice we have found the leakage from this source is practically *nil.*

The small door in the back, measuring one foot square, is so arranged that one may paste paper or any fabric over the opening in such a way as to determine its permeability to a given fumigating agent. It is frequently necessary to do this, particularly if one wishes to know the value of such material when employed for closing

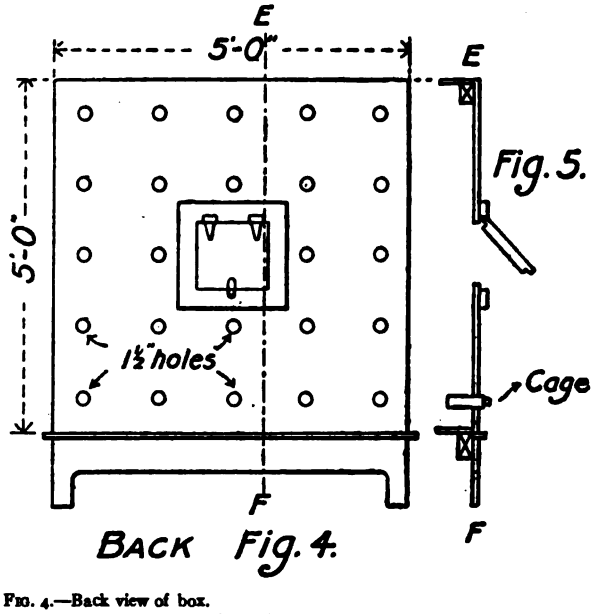


FIG. 4.—Back view of box.
FIG. 5.—Closing device for door; the overlapping is the same as the front door.

openings in buildings which are to be fumigated. The function of the glass windows, each one foot square, upon the sides, has already been commented upon.

Holes measuring one and one-half inches in diameter are bored at regular intervals through the bottom, top, sides, front, and back of the box. Particular attention is paid to have the alignment with reference to the height above the floor exact, because the distance above the floor is a very important factor to consider with many fumigating substances, particularly if the fumes are light, and consequently very dense at the top of the room, but practically

absent at the bottom. For this reason holes are bored in the floor, so that mosquitoes may be introduced and exposed at the point of minimum efficiency of the fumigant.

The cages are composed of wire netting of a mesh not less than 20 to the inch. This is extremely important; repeatedly the writer has seen *Stegomyia fasciata* pass through a 17–18 mesh netting. One end is closed with a circular piece of the same material, the seam soldered, and the other end provided with a band of tin, one inch wide, which fits inside the cylinder, serving the twofold purpose of keeping the cylinder in shape at this end and furnishing a point of attachment for the wooden stoppers.

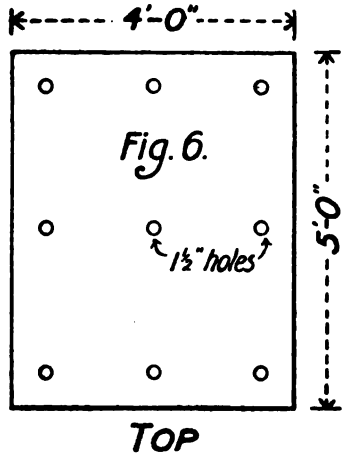


FIG. 6.—Top of box.

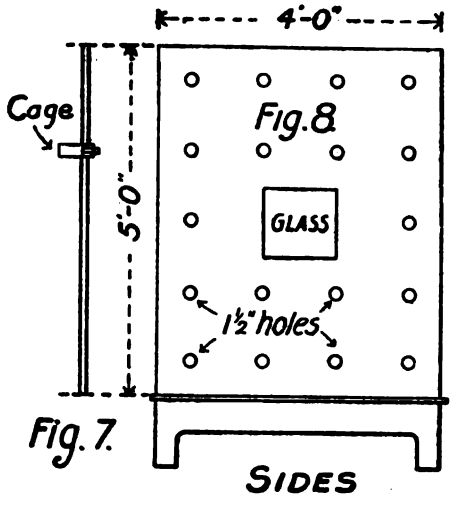
The latter are tapered so that they fit tightly inside the tin lining of the wire cage, and at the same time fit tightly into the hole in the side of the box through which the wire cage just passes. A half-inch hole through the long axis of the wooden stopper permits the introduction of mosquitoes, and a cork stopper of appropriate size closes this hole when the mosquitoes are in place, preventing loss of mosquitoes or fumes.

A collection of wooden stoppers which fit the holes in the sides of the box, and which serve to close these openings when they are not occupied by mosquito cages, completes the outfit.

The writer has not only tested comparatively the ordinary fumigating agents, but has made a series of careful studies to show the relation of results obtained under ideal conditions as initiated in the apparatus above described with those obtained in large buildings. The great amount of fumigating which is being done in Panama permits such comparisons on a practical scale, and, without going too much into detail, the results show in general that a slightly larger amount of fumigating agent per unit space is required in the relatively small box than in a building which can be closed tightly. This seems to be due, in part at least, to the proportionately large amount

of surface in the box as compared with that of a very large room. The absorption and surface condensation are increased as the surface increases, and a smaller room, of course, has relatively a larger surface than a larger room of the same general shape.

Hence, as a rule, the fact that mosquitoes are killed in this apparatus with a certain amount of fumigant per unit space, under given conditions, will hold for a larger room, under the same conditions.



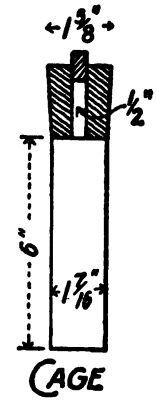


FIG. 9.

FIG. 7.—Section of side showing one cage in position; the cage projects into the lumen of the box and is closed on the outside by a small stopper.

FIG. 8.—Sides of box.

FIG. 9.—One of the wire-gauze cages showing wooden stopper, with hole for introduction of mosquitoes closed by a wooden stopper.

Conversely, and even more certainly, if a given concentration of fumigant *will not* kill in the experimental box, in a large space the fumigation, under the same conditions, will be unsuccessful.

A very interesting and important point has come up in connection with this work; namely, the rapidity with which the active fumigating agent is evolved makes a great deal of difference in the efficiency of the fumigation. For example, with the less active fumigants and culicides, as pyrethrum, the difference between complete success and complete failure may depend upon the rapidity with which the culicidal substance is evolved as smoke. One pound per 1,000 feet burned in three portions simultaneously is rather more

efficient than one and one-half pounds burned in one lot. This seems to hold true to a lesser extent with all the culicides.

The amount of furniture, and in general of objects which occupy much space, diminishes the efficiency of the culicidal action, and should be taken into account in practical as well as experimental fumigations.

THE EFFECT OF SUBCUTANEOUS INJECTIONS OF WATER, RINGER'S FLUID, AND TEN PER CENT SOLUTIONS OF ETHYL ALCOHOL UPON THE COURSE OF FATIGUE IN THE EXCISED MUSCLES OF THE FROG.*

THEODORE HOUGH AND CLARA ELEANOR HAM

(*From the Biological Laboratories of the Massachusetts Institute of Technology.*)

THE research, the results of which are herewith reported, was suggested by a paper by Lee and Salant[1] on the effect of alcohol upon the fatigue of skeletal muscle. These investigators, after ligaturing one leg of a frog near the hip joint, injected a 10 per cent solution of ethyl alcohol into the dorsal lymph sac or the stomach; the ligatured (normal) leg was at once removed below the ligature, and a fatigue tracing taken from the gastrocnemius, using isotonic contractions and giving about 60 stimuli a minute. After allowing from 20 to 75 minutes for the absorption of the injected fluid, a similar tracing was taken from the "alcoholized gastrocnemius of the other leg. Thus from each animal records were obtained from a non-alcoholized and an alcoholized muscle."

The results of a large number of experiments made by this very ingenious method are thus summarized by the authors: Tracings from the alcoholized muscle showed "quicker contraction, quicker relaxation, larger number of contractions and increase of work in a given time, larger number of contractions and greater total amount of work before exhaustion sets in, and delay of fatigue;" and the conclusion is drawn that "in medium quantity it (i. e., ethyl alcohol) exerts a favorable action" upon skeletal muscle.

We can fully corroborate Lee and Salant's statement that the "alcoholized" muscle contracts and relaxes more quickly than the

* Received for publication April 13, 1906.

[1] LEE AND SALANT, *Amer. Jour. of Physiol.*, 1902, 8, pp. 61–74.

muscle of the ligatured leg. This difference is seen in the earlier contractions of the series and becomes more pronounced in the later contractions. The contraction time of the 100th twitch of the normal muscle, indeed, may exceed one second, while that of the alcoholized muscle may not exceed a fifth of a second; and this is specially true of the period of relaxation, which is often disproportionally lengthened in the "normal" muscle.

With regard to the statement that the alcoholized muscle gives "a larger number of contractions," we have found that a larger number of *complete* simple contractions (i. e., twitches in which relaxation is complete before the next contraction begins) may be obtained from the alcoholized muscle. But it would seem that Lee and Salant have taken the disappearance of individual simple contractions as an indication of the exhaustion of the muscle. Figs. 1 and 4 of their paper, however, show clearly that, while the individual contractions of the normal muscle do indeed cease sooner than those of the alcoholized muscle, this is due to the fact that relaxation is not complete and *the muscle has gone into tetanus.* Obviously, such a tracing does not show exhaustion.

In order to eliminate this disturbing factor of increased relaxation time, we have repeated the experiments with the exception that in one series less rapid rates of stimulation (one every two or three seconds) were used, and in another series the two muscles were thrown into tetanus and their tracings compared. In all cases the alcoholized muscles showed greater resistance to fatigue; and during the tetanic contractions the weight was not only lifted to a greater height, but also held at a greater height than by the "normal" muscle.

Lee and Salant's results as to the behavior of these "alcoholized" muscles were, therefore, confirmed in all essential points. After the absorption of the 10 per cent alcohol the muscle did more work, and the onset of fatigue was distinctly delayed.

In all these experiments 0.08 c.c. of the 10 per cent alcohol per gram of body-weight were injected. This means, for a medium-sized frog, the addition of 2.5 c.c., or more, of fluid to the circulating medium of the body—by no means an inconsiderable quantity in this animal. Consequently it is still an open question whether the favor-

able effect upon the working power of the muscle is to be attributed to the ethyl alcohol, or to the increase of the circulating medium.

In order to test this, we made parallel, simultaneous experiments, injecting into one frog the solution of alcohol, and into another an equivalent amount of water, or Ringer's fluid. The operative procedure and experimental method were essentially those of Lee and Salant: After removal of the cerebrum, with due precautions against loss of blood, one leg of each animal was ligatured and the fluid injected into the dorsal lymph sac. Simultaneous tracings were then taken from the excised muscles of the ligatured legs; and later (usually about 45 minutes), simultaneous tracings were taken from the other legs. Edison-Lalande cells were used to insure constancy in the strength of the stimulating current, the primary circuit being interrupted, and the "making" induction shock short circuited by a mechanical "Ablender." The rate of stimulation was usually about once every two seconds. In some experiments the contractions were isotonic, the same weight being lifted by the two muscles of the same frog. In other experiments the auxotonic method was employed, the muscle contracting against the resistance of an open, spiral, brass spring, accurately adjusted so as to insure equal initial tension and the same direction of pull.

The work done during the same time by two muscles, in fatigue tracings taken on drums revolving at the same, uniform speed, and with the same rate of stimulation, is proportional to the areas included between the base line,* the first and last tracings of the given period, and the line joining the highest points of the contraction records. These areas, were therefore measured with a planimeter, and the work done by the two muscles readily compared.

RESULTS.

Thirteen successful experiments were thus made upon the effects of injecting the 10 per cent alcohol, and 13 simultaneous, parallel experiments upon the effects of injecting water, or Ringer's fluid. The results with each of these fluids are given separately, for convenience, and the percentage of increase of work done in the same time (until the appearance of marked fatigue) is represented by giving the number of experiments in which a given range of increase occurred.

*In our experiments the contractions returned to the base line.

TABLE 1.

THE EFFECT OF INJECTING 10 PER CENT ALCOHOL.

Percentage Increase of Work	Number of Experiments
0–10	4
11–20	2
21–30	4
31–40	2
——	—
——	—
100–110	1
Total	13

TABLE 2.

THE EFFECT OF INJECTING RINGER'S FLUID.

Percentage Increase of Work	Number of Experiments
0–10	1
11–20	0
21–30	1
31–40	1
41–50	0
51–60	1
61–70	1
Total	5

TABLE 3.

THE EFFECT OF INJECTING WATER.

Percentage Increase of Work	Number of Experiments
0–10	0
11–20	0
21–30	2
31–40	1
41–50	2
——	—
——	—
191–200	1
Total	6

It is evident that the injection of water and of Ringer's fluid, as well as that of the 10 per cent alcohol, was followed by an increase of work done. The experiments are not sufficiently numerous to justify final conclusions as to the relative influence of the alcohol as against the water, or Ringer's fluid; but it is significant that the percentage of increase is less after the injections of alcohol than after the injections of the other fluids. Thus while 12 out of 13 alcohol experiments gave less than 40 per cent of increase, 5 of 13 with water and Ringer's fluid gave more than 40 per cent. Moreover, the increase in six of the alcohol experiments was less than 20 per cent, while in

only one of the others was it as small as this. The conclusion is certainly justified that the effect of the injections of 10 per cent alcohol is not due to the alcohol, but to the water in which it is dissolved; and it is even probable that the increase of work occurred *in spite* of the presence of the alcohol, rather than *because* of it.

THE EFFECT OF DOUBLE INJECTIONS.

In order to test still further the conclusions stated in the last paragraph, we have injected, in a certain number of animals, water or Ringer's fluid one or more hours before ligaturing the leg; the subsequent procedure was the same as in other experiments—ligature of one leg, followed by a fatigue tracing therefrom; then a (second) injection, this time of water or 10 per cent alcohol; and, an hour later, a fatigue tracing from the second leg. We desired to find whether the second injection of 10 per cent alcohol would be without effect, or show a less marked effect after the previous injection of water; and, also, to find how the effect of alcohol would compare with those of a second injection of water. The results are as follows:

a) *Water followed by water.*—Three experiments, which gave increases of 26.7 per cent, 6 per cent, and 1 per cent of work by the second muscle over the work of the first.

b) *Water followed by 10 per cent alcohol.*—Three experiments, giving increases of 25.4 per cent, 6.9 per cent, and 6.5 per cent.

c) *Ringer's fluid followed by alcohol.*—One experiment, giving an increase of 11.1 per cent.

d) *Water followed by water.*—Two experiments with auxotonic contractions, giving in one case an increase of 7.3 per cent, and in the other a decrease of 18 per cent.

The effect of the second injection of water or alcohol is, therefore, much less marked when it follows a previous injection of water. Obviously, we should expect this result, if the effect in all cases is due to the addition of water to the circulating medium, or to the improvement of the circulation by increasing the volume of blood.

All the experiments of Lee and Salant were made between January and June, and, therefore, like most laboratory experiments upon this animal, were made upon winter frogs, which have been inactive for several months, and in which the loss of water from the blood, by the

processes of excretion, has not been made good by the taking of food. Under these circumstances, the injection of any fluid quickly increases the volume of blood, and so improves the circulation through all organs—the muscles included. Perhaps, also, by reducing the osmotic tension of the plasma, waste products which have accumulated in the muscle during the inactive winter period are removed, so that the excised muscle becomes capable of greater work. This obviously suggests a useful method of improving the physiological condition of frogs used for laboratory experiments during the winter months.

SUMMARY.

1. Injections of water, Ringer's fluid, and 10 per cent ethyl alcohol into the lymph sacs of a frog improves the working capacity of the skeletal muscles and delays the progress of fatigue in the isolated muscle.

2. The effect is largely independent of the solution and seems to be due to the increase of the circulating medium, or to the reduction of osmotic tension in the blood plasma, or to both of these causes combined.

3. The improvement in the working power of the muscle, which Lee and Salant observed after injections of 10 per cent alcohol, is not due to the alcohol.

NOTES ON A CASE OF APPARENT PULMONARY TUBERCULOSIS ASSOCIATED WITH THE CONSTANT PRESENCE OF DIPHTHERIA-LIKE ORGANISMS IN THE SPUTUM.*

BURT RANSOM RICKARDS,
Director, Boston Board of Health Bacteriological Laboratory.

ON January 8, 1906, a sample of sputum from H——S—— was submitted to this laboratory in one of the regular outfits to be examined for the bacilli of tuberculosis. In the course of the regular routine the specimen was stained in steaming carbol-fuchsin for five minutes, decolorized with acid-alcohol (5 per cent HCl), and counterstained with Loeffler's methylene blue. When examined under the microscope, no bacilli of tuberculosis were found, but the specimen contained large numbers of bacilli which morphologically were indistinguishable from the bacillus of diphtheria. In fact, no other species of organisms were apparent in the specimen, microscopically. As is usual in such cases, the attending physician was requested to secure a second, and in this case uncarbolized, specimen. The second specimen was received on January 15, 1906, and from it the organisms in question were isolated, but not without some difficulty, owing to the presence of a large, rapidly growing coccus which tended to spread and overgrow the diphtheria-like organism. The cultural characteristics of the organism were then studied as follows:

Morphology.—Indistinguishable from the Klebs-Loeffler organism. A, C, and D types (Wesbrook) present; C types predominating. No spores. Stains by the ordinary stains, and by Gram's and Neisser's stain.

Hanging-block.—The post-fission movement called "snapping," and which appears to be characteristic of the diphtheroid group, was here observed.[1]

Agar stroke.—Filiform growth at first, later slightly plumose; elevation fla with slightly raised edges. Luster glistening. Topography smooth at first, later slightly contoured. Opaque, non-chromogenic. Consistency, butyric. Medium not discolored.

*Received for publication April 7, 1906.

[1]HILL AND RICKARDS. *Rep. and Papers, Amer. Pub. Health Assoc.*, 28, p. 479.

Potato.—Grows well, but is invisible.

Blood serum.—Growth as described under agar stroke, except that it grows more luxuriantly, spreads slightly, and is faintly flesh-colored. Typical appearance.

Agar stab.—Filiform, spreading slightly on surface.

Gelatin stab.—Filiform; no liquefaction; slight growth on surface.

Broth.—Surface growth, none; tubidity, slight; sediment, considerable, granular —adhering to glass.

Milk.—Coagulation, none; consistency, unchanged.

Agar colonies.—Punctiform; edge entire; finely granular.

Relative growth at 20° and 37°.—Grows well at 37° C.; very slow growth at 20° C.

*Fermentation of dextrose.**—No gas production; no growth in closed arm; acidifying coefficient: 1 day, $\frac{1.1}{0.5}$; 2 days, $\frac{2.0}{0.5}$; 4 days, $\frac{3.00}{0.5}$; 6 days, $\frac{3.2}{0.5}$; 8 days, $\frac{3.4}{0.5}$; 10 days, $\frac{3.2}{0.5}$.

*Fermentation of lactose.**—No acid or gas production.

*Fermentation of saccharose.**—No acid or gas production.

Indol production.—Very slight.

Pathogenesis.—Very slight. A guinea-pig inoculated with 1 per cent of his body-weight of a 0.2 per cent dextrose broth grown 10 days at 37° C. showed a very slight edema at site of inoculation and slight injection of suprarenals in two days.

For the following clinical history the writer is indebted to Dr. A. H. Bassett the attending physician:

Patient, Miss H— S—. Age, 32 years; medium height; sandy complexion; slightly stoop-shouldered; weight, 112 pounds. Occupation, clerk. First consulted physician on December 30, 1905. Symptoms: loss of flesh, pain in upper chest and right shoulder, loss of appetite.

Present illness dates back five years, previous to which patient said she had no sickness since childhood. No history of diphtheria. First symptoms were decided hoarseness, aggravated by dampness or by becoming over-tired. For past two years the amount of perspiration has been over normal. Regular night sweats eight months ago; not as profuse at present time. Took care of consumptive sister one year ago. Symptoms gradually growing worse since then. Some expectoration, but not abundant. Within the past year urine has at times contained some blood. This condition lasts for some time and then passes off. The patient's urine was examined by one physician who reported "nothing pathologic." No urine analysis by writer.

Physical examination (reported by Dr. H. C. Clapp): "Small area of dulness in apex of right lung, heard more posteriorily; broncho-vesicular respiration; bronchophony; normal temperature. Clinically, tuberculosis."

On February 26, six weeks after the last previous sputum was examined, a third specimen was requested and obtained, in order to demonstrate the continued presence of the diphtheria-like organism. No tubercle bacilli were found, but the above organism was present in undiminished numbers.

* 0.2 per cent. in sugar free-broth.

PLATE 3.

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Three separate examinations have thus been made on three samples of sputum submitted at intervals, and in each case no tubercle bacilli were found after an exhaustive search. This fact in itself does not, of course, rule out the possibility, but lessens by so much the probability, of their presence in the lung tissue. On each of these examinations an organism resembling *B. diphtheriae* both morphologically and culturally has been present in large numbers.

The fact of the continued presence of this organism raises a suspicion in the mind of the writer as to whether the organism, if not the primary, may not at least be a contributing factor in producing the symptoms above described.

Dr. Louis Hoag, of the Danvers, Mass., State Hospital, has in a number of cases isolated diphtheria-like organisms from the lungs of patients who had died of broncho-pneumonia. Dr. Hoag agrees with the writer that these organisms differ in many respects from the one here described.

The writer is unaware of any previous descriptions in the literature analogous to the above. Dr. W. H. Smith, of this city, has, however, very kindly submitted notes on a case which came under his care at the Massachusetts General Hospital in 1904. The clinical history bears a most decided resemblance, including a family history of tuberculosis, tubercular symptoms, cough—four years—moist râles, apex of right lung, etc. Sputum examinations show no bacilli of tuberculosis, but the constant presence of an organism resembling the diphtheria bacillus morphologically, but of very low virulence.

It is the intention of the writer to follow the two cases cited, if circumstances permit, with a view to determining, if possible, the rôle played by the diphtheria-like organisms.

The writer wishes to acknowledge his indebtedness to Dr. F. H. Slack, Assistant Bacteriologist, for help in isolating and identifying the organism.

DESCRIPTION OF PLATE 3.

Pure culture, diptheria-like, organism isolated from sputum. Twenty-four-hour growth in serum. Methylene blue. × 1500.

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